VERIFICATION OF A TRANSLATION

I, the below named translator, hereby declare that:

My name and post office address are as stated below:

That I am knowledgeable in the English language and in the language in which the below identified international application was filed, and that I believe the English translation of the international application No. PCT/JP00/04837 is a true and complete translation of the above identified international application as filed.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date

December 19, 2001

Full name of the translator Yumi KUJIME

Signature of the translator Yumi Kujime

Post Office Address Kitahama TNK Building 7-1, Dosho-machi

1-chome, Chuo-ku, Osaka-shi, Osaka 541-0045,

Japan

ATENT COOPERATION TF TY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 30 March 2001 (30.03.01)	
International application No. PCT/JP00/04837	**Applicant's or agent's file reference** P00-14
International filing date (day/month/year) 19 July 2000 (19.07.00)	**Priority date** (day/month/year) 21 July 1999 (21.07.99)
Applicant OZEKI, Yoshihiro et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

16 February 2001 (16.02.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Maria Kirchner
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

US

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
P00-14		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/JP 00/ 04837	19/07/2000	21/07/1999
Applicant		
SAN-EI GEN F.F.I., INC. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [X] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

MITES-LIKE ELEMENT AND TRANSCRIPTIONAL ACTIVATION ELEMENT

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP 00/04837

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-5, 10-12, and partially 8, 9 and 13-19

   MITE-like element capable of causing duplication of the target sequence (A)nG(A)n; transcriptional activation element containing it; transgene expression cassette and plasmid containing the same and transgenic plant containing such an expression cassette

2. Claims: 6 and 7, and partially 8, 9 and 13-19

   MITE-like element capable of causing duplication of the target sequence TA, comprising SEQ ID NO:6 and showing SEQ ID NO:4 in the 5' terminal region and SEQ ID NO:5 in the 3' terminal region; transcriptional activation element containing it; transgene expression cassette and plasmid containing the same and transgenic plant containing such an expression cassette

3. Claims: partially 8, 9 and 13-19

   Transcriptional activation element containing a transposable element other than a MITE-like element under subject 1 and 2; transgene expression cassette and plasmid containing the same and transgenic plant containing such an expression cassette

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]/JP 00/04837

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/90 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBL, WPI Data, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BUREAU T E ET AL: "Stowaway: a new family of inverted repeat elements associated with the genes of both monocotyledonous and dicotyledonous plants" PLANT CELL,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD,US, vol. 6, no. 6, June 1994 (1994-06), pages 907-916, XP002156106 ISSN: 1040-4651 cited in the application the whole document --- -/--	1-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 April 2001	09. 05. 2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BÉNÉDICTE CHARRIER ET AL.: "Bigfoot: a new family of MITE elements characterized from the Medicago genus" PLANT JOURNAL., vol. 18, no. 4, May 1999 (1999-05), pages 431-441, XP002157570 BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD., GB ISSN: 0960-7412 the whole document	1-5,9-19
A	ELENA CASACUBERTA ET AL.: "Presence of miniature inverted-repeat transposable elements (MITEs) in the genome of Arabidopsis thaliana: characterisation of the Emigrant family of elements" PLANT JOURNAL., vol. 16, no. 1, October 1998 (1998-10), pages 79-85, XP002156103 BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD., GB ISSN: 0960-7412 the whole document	1-5,9-19
X	WO 98 22593 A (PIONEER HI-BRED INTERNATIONAL, INC.) 28 May 1998 (1998-05-28) page 6, line 27 -page 7, line 17 page 7, line 31 -page 8, line 7; examples 1,2	8,13-19
X	SHIN TAKEDA ET AL.: "Transcriptional activation of the tobacco retrotransposon Tto1 by wounding and methyl jasmonate" PLANT MOLECULAR BIOLOGY, vol. 36, 1998, pages 365-376, XP002165578 abstract page 370, left-hand column, paragraph 1 -right-hand column, paragraph 2 page 373, right-hand column, paragraph 2 -page 374, right-hand column, paragraph 2	8,13-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
/JP 00/04837

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9822593 A	28-05-1998	US 5955361 A	21-09-1999
		AU 5455498 A	10-06-1998

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P00-14		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/JP00/04837	19/07/2000	21/07/1999
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant SAN-EI GEN F.F.I., INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
16/02/2001	23.10.2001
Name and mailing address of the international preliminary examining authority: European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized officer Montero Lopez, B Telephone No. +31 70 340 3739

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/JP00/04837

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-78 as originally filed

Claims, No.:

1-19 as originally filed

Drawings, sheets:

1/13-13/13 as originally filed

Sequence listing part of the description, pages:

1-7, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

   ☐ restricted the claims.

   ☒ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

   ☐ complied with.

   ☒ not complied with for the following reasons:
   see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

   ☒ all parts.

   ☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

   Novelty (N) Yes: Claims 1-7, 9-12

	No:	Claims	8, 13-19
Inventive step (IS)	Yes:	Claims	1-7, 9-12
	No:	Claims	8, 13-19
Industrial applicability (IA)	Yes:	Claims	1-19
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item IV

Lack of unity of invention

The present application relates to miniature inverted-repeat transposable element (MITE)-like elements obtained from carrot and uses thereof. Multiple MITE-like elements have been identified already in plants and disclosed in the prior art. The article "The Plant Cell" 1994, vol. 6, pages 907-916 describes for instance the Stowaway family of MITE-like elements identified in monocotyledonous and dicotyledonous plants. "The Plant Journal" 1998, vol. 16, no. 1, pages 79-85 describes the Arabidopsis thaliana Emigrant family of MITE-elements and shows evidence for their mobility. In the light of the prior art a problem underlying the present application can be formulated as providing further MITE-like elements. The following solutions are proposed in the claims:

1. MITE-like element capable of causing duplication of the target sequence (A)nG(A)n.

2. MITE-like element capable of causing duplication of the target sequence TA and comprising SEQ ID NO:6.

A further problem identified in the present application consists in the provision of a transcriptional activation element which avoids inactivation of the inserted gene. The solution proposed is as follows:

3. A transcriptional activation element comprising at least a transposable element.

Given the essential difference between the sequences of the polynucleotides provided as solutions to the first problem, due to the fact that plant MITE-like elements have already been disclosed in the state of the art, and since in the light of the state of the art, no other technical feature could be distinguished as being new and common to the proposed problems and solutions, the IPEA is of the opinion that there is no single inventive concept underlying the plurality of the claimed inventions in the present application, in the sense of Rule 13.1 PCT and Article 34(3)(a) PCT.

As two extra examination fees have been paid the international examination report is established for all the inventions mentioned in the claims (see paragraphs 1, 2 and 3

above).

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: BUREAU T E ET AL: 'Stowaway: a new family of inverted repeat elements associated with the genes of both monocotyledonous and dicotyledonous plants' PLANT CELL,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD,US, vol. 6, no. 6, June 1994 (1994-06), pages 907-916, XP002156106 ISSN: 1040-4651 cited in the application

D2: ELENA CASACUBERTA ET AL.: 'Presence of miniature inverted-repeat transposable elements (MITEs) in the genome of Arabidopsis thaliana: characterisation of the Emigrant family of elements' PLANT JOURNAL., vol. 16, no. 1, October 1998 (1998-10), pages 79-85, XP002156103 BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD., GB ISSN: 0960-7412

D3: WO 98 22593 A (PIONEER HI-BRED INTERNATIONAL, INC.) 28 May 1998 (1998-05-28)

1. Claims 1-5, 10-12 and partially claims 8, 9 and 13-19 relate to a MITE-like element capable of causing duplication of the target sequence (A)nG(A)n. Claims 6 and 7 and partially claims 13-19 relate to a MITE-like element capable of causing duplication of the target sequence TA and comprising SEQ ID NO:6. No such MITE-like elements have been described in the state of the art and therefore claims 1-7, 10-12 and claims 8, 9 and 13-19 as far as depending on these are novel according to Article 33(2) PCT.

1.1. Documents D1 and D2, which are considered to represent the most relevant state of the art, disclose, respectively, the Stowaway family of MITE-like elements identified in monocotyledonous and dicotyledonous plants and the Arabidopsis thaliana Emigrant family of MITE-elements and evidence for their mobility. In the

light of the prior art a problem underlying the present application can be formulated as providing further MITE-like elements. The solutions provided by the application, that is a MITE-like element capable of causing duplication of the target sequence (A)nG(A)n and a MITE-like element capable of causing duplication of the target sequence TA and comprising SEQ ID NO:6 have never been suggested in the prior art. Therefore, the skilled person would not have been able to provide such MITE-like elements without exercising an inventive step. Claims 1-7, 10-12 and claims 8, 9 and 13-19, as far as depending on these involve an inventive step and comply with the requirements of Article 33(3) PCT.

2. Claims 8, 9 and 13-19 encompass as well a transcriptional activation element containing a transposable element other than as disclosed under claims 1-7 and 10-12.

2.1. Document D3 discloses a transcriptional regulatory region of a gene for driving expression of a gene in a plant (see page 6, line 27 - page 7, line 15) and a vector comprising the same (see page 7, line 31 - page 8, line 7) for generating transgenic plants including the P gene promoter in which the transposable element Ac has been inserted. In the light of D3, claims 8 and 13-19 are therefore not novel and do not comply with the requirements of Article 33(2) PCT.

2.2 Claim 9 specifies the transposable element as a MITE-like element. Since the transposable element mentioned in D3 is not a MITE-like element, claim 9 is novel and meets the requirements of Article 33(2) PCT.

2.3. In the light of the state of the art, the problem to be solved consist in the provision of a transcriptional regulatory region as disclosed in D3 comprising a transposable element other than Ac. Document D2 discloses the Arabidopsis thaliana Emigrant family of MITE-elements and provides evidence for their mobility. However, no hint in the state of the art exists which would prompt the skilled person to replace the Ac transposable element of the construct disclosed in D3 by the MITE-element disclosed in D2. Claim 9 is therefore considered to involve an inventive step according to Article 33(3) PCT.

Re Item VII

Certain defects in the international application

1. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the document D3 is not mentioned in the description, nor is this document identified therein.

2. The term "coding" in claims 10 and 11, paragraphs (b) and (d) is used in a different sense than its normal meaning in the art (nucleotide sequence coding for an amino acid sequence).

Re Item VIII

Certain observations on the international application

1. Claim 1 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The claim attempts to define the subject-matter in terms of the result to be achieved which merely amounts to a statement of the underlying problem, that is providing a MITE-like element capable of causing duplication of the target sequence $(A)_nG(A)_n$. The technical features necessary for achieving this result should be added.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
SAEGUSA, Eiji
Kitahama TNK Building
1-7-1, Doshomachi, Chuo-ku
Osaka-shi, Osaka 541-0045
JAPON

Date of mailing (day/month/year) 25 January 2001 (25.01.01)		
Applicant's or agent's file reference P00-14	**IMPORTANT NOTICE**	
International application No. PCT/JP00/04837	**International filing date** (day/month/year) 19 July 2000 (19.07.00)	**Priority date** (day/month/year) 21 July 1999 (21.07.99)
Applicant SAN-EI GEN F.F.I., INC. et al		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AG,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CR,CU,CZ,DE,DK,DM,DZ,EA,EE,EP,ES,
   FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,
   MW,MX,MZ,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   25 January 2001 (25.01.01) under No. WO 01/05986

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

INTERNATIONAL SEARCH REPORT

[redacted]onal Application No
[redacted]T/JP 00/04837

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/90 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBL, WPI Data, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BUREAU T E ET AL: "Stowaway: a new family of inverted repeat elements associated with the genes of both monocotyledonous and dicotyledonous plants" PLANT CELL,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD,US, vol. 6, no. 6, June 1994 (1994-06), pages 907-916, XP002156106 ISSN: 1040-4651 cited in the application the whole document --- -/--	1-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 April 2001	09. 05. 2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BÉNÉDICTE CHARRIER ET AL.: "Bigfoot: a new family of MITE elements characterized from the Medicago genus" PLANT JOURNAL., vol. 18, no. 4, May 1999 (1999-05), pages 431-441, XP002157570 BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD., GB ISSN: 0960-7412 the whole document	1-5,9-19
A	ELENA CASACUBERTA ET AL.: "Presence of miniature inverted-repeat transposable elements (MITEs) in the genome of Arabidopsis thaliana: characterisation of the Emigrant family of elements" PLANT JOURNAL., vol. 16, no. 1, October 1998 (1998-10), pages 79-85, XP002156103 BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD., GB ISSN: 0960-7412 the whole document	1-5,9-19
X	WO 98 22593 A (PIONEER HI-BRED INTERNATIONAL, INC.) 28 May 1998 (1998-05-28) page 6, line 27 -page 7, line 17 page 7, line 31 -page 8, line 7; examples 1,2	8,13-19
X	SHIN TAKEDA ET AL.: "Transcriptional activation of the tobacco retrotransposon Ttol by wounding and methyl jasmonate" PLANT MOLECULAR BIOLOGY, vol. 36, 1998, pages 365-376, XP002165578 abstract page 370, left-hand column, paragraph 1 -right-hand column, paragraph 2 page 373, right-hand column, paragraph 2 -page 374, right-hand column, paragraph 2	8,13-19

2

INTERNATIONAL SEARCH REPORT

[redacted]ation on patent family members

[redacted].onal Application No
[redacted]T/JP 00/04837

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9822593 A	28-05-1998	US 5955361 A	21-09-1999
		AU 5455498 A	10-06-1998

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP 00/04837

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-5, 10-12, and partially 8, 9 and 13-19

   MITE-like element capable of causing duplication of the target sequence (A)nG(A)n; transcriptional activation element containing it; transgene expression cassette and plasmid containing the same and transgenic plant containing such an expression cassette

2. Claims: 6 and 7, and partially 8, 9 and 13-19

   MITE-like element capable of causing duplication of the target sequence TA, comprising SEQ ID NO:6 and showing SEQ ID NO:4 in the 5' terminal region and SEQ ID NO:5 in the 3' terminal region; transcriptional activation element containing it; transgene expression cassette and plasmid containing the same and transgenic plant containing such an expression cassette

3. Claims: partially 8, 9 and 13-19

   Transcriptional activation element containing a transposable element other than a MITE-like element under subject 1 and 2; transgene expression cassette and plasmid containing the same and transgenic plant containing such an expression cassette

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant *(for all designated States except US)*: SAN-EI GEN F.F.I., INC. [JP/JP]; 1-11, Sanwa-cho 1-chome, Toyonaka-shi, Osaka 561-0828 (JP).

(71) Applicant and
(72) Inventor: OZEKI, Yoshihiro [JP/JP]; 2-3-6-302, Daimon-cho, Higashikurume-shi, Tokyo 203-0011 (JP).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: OYANAGI, Mikiko [JP/JP]; 2-6-1-1007, Midori-cho, Fuchu-shi, Tokyo 183-0006 (JP). FUKUDA, Takashi [JP/JP]; 612-8, Kamimizo, Sagamihara-shi, Kanagawa 229-1123 (JP). KODA, Takatoshi [JP/JP]; San-Ei Gen F.F.I., Inc., 1-11, Sanwa-cho 1-chome, Toyonaka-shi, Osaka 561-0828 (JP).

(74) Agents: SAEGUSA, Eiji et al.; Kitahama TNK Building, 1-7-1, Doshomachi, Chuo-ku, Osaka-shi, Osaka 541-0045 (JP).

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(54) Title: NOVEL MINIATURE INVERTED-REPEAT TRANSPOSABLE ELEMENTS (MITEs)-LIKE ELEMENT AND TRANSCRIPTIONAL ACTIVATION ELEMENT

(57) Abstract: The invention provides novel, carrot-derived MITE-like elements (transposable elements). It further provides transcriptional activation elements comprising at least one transposable element, in particular one of the above MITE-like elements. Specifically, it provides transcriptional activation elements having a DNA comprising the nucleotide sequence shown under SEQ ID NO:1 or a functional equivalent thereto and/or a DNA comprising the nucleotide sequence shown under SEQ ID NO:2 or a functional equivalent thereto. The transcriptional activation elements of the invention can increase or activate the reduced expression of a foreign gene introduced by the transgenic technology. Therefore, the transcriptional activation elements contribute to stable expression of a foreign gene introduced in a plant genom and are useful in stably producing genetically modified plants.

DESCRIPTION

NOVEL MINIATURE INVERTED-REPEAT TRANSPOSABLE ELEMENTS (MITEs)-LIKE ELEMENT AND TRANSCRIPTIONAL ACTIVATION ELEMENT

TECHNICAL FIELD

The present invention relates to novel, plant-derived transposable elements and, more particularly, to a MITE (miniature inverted-repeat transposable element)-like sequence. More specifically, the present invention relates to novel transposable elements isolated from carrot (*Daucus carota*).

The invention further relates to novel transcriptional activation elements containing the transposable elements. More specifically, it relates to a transcriptional activation element which, when inserted in a gene, is capable of promoting the transcription of a gene around or in the vicinity of the site of insertion thereof or suppressing the transcription of said gene from being inactivated. The invention still further relates to a transgenic plant harboring said transcriptional activation element.

BACKGROUND ART

Transposable elements are found in the genomes of almost all living organisms, without distinction between prokaryotes and eukaryotes, or between animals and plants. It is known that these transposable elements can move from a genomic gene to another and inactivate the genomic gene upstream or downstream from the sequence in which such an element has been inserted. Further, it has been revealed that, in addition to such actions, transposable elements cause various types of genomic reorganization (deletion, inversion, duplication, etc.) and it has been reported that the above fact leads to genome plasticity, which is important for the evolution of living organisms and, in particular, that transposable elements play an important role in the evolution of living organisms for environmental adaptation in response to genomic stresses (McClintock, Science 26: 792-801 (1984); Arber et al., FEMS Microb. Ecol., 15: 5-14 (1994)).

Transposable elements are roughly classified into three types: DNA type (transposons and insertion sequences), RNA type (retrotransposons) (Berg et al. (ed.), Mobile DNA (1989)), and miniature inverted-repeat transposable lements (MITEs)

belonging to neither of the above two types (Wessler et al., Curr. Opin. Genet. Dev. 5: 914-821 (1995)).

As for DNA type and RNA type transposable elements among them, their actual transposition in the genome has been established whereas, for MITEs, no reports have so far been made about evidences of their transposition in the genome in spite of their being very similar to DNA type ones.

Most of MITEs have been found out, by computer retrieval, from databases of nucleotide sequences of genomic genes of various living organisms as being elucidated by genome projects currently in progress. The first discovery was the discovery in 1992 of the *Tourist* family by Bureau et al. (Bureau et al., Plant Cell 4: 1283-1294 (1992)). Since then, various MITEs have been found in plant genomes and in insect and animal genomes.

Generally, MITEs are defined as having such characteristics as (1) their having a perfect or imperfect inverted repeat sequence at each of both the 5'- and 3'-terminal regions (similar in this respect to DNA type transposable elements), (2) occurrence of a target duplication-like sequence, of a sequence consisting of two or more nuclueotides, like the one f rmed upon insertion of a DNA type

transposable element into a genomic DNA, on both the terminal sides of the inverted repeat sequences, and (3) their generally having a size of shorter than 2 kb (Wessler et al., Curr. Opin. Genet. Dev. 5: 914-821 (1995)).

As those MITEs which have an open reading frame coding for a transposase between both the terminal inverted repeat sequences, like DNA type transposable elements, there are known only MITEs of the IS630-Tc1/Mariner family found in molds and animals (Kachroo et al., Mol. Gen. Genet. 245: 339-348 (1994); Smit et al., Proc. Natl. Acad. Sci. USA 93: 1443-1448 (1996)). As far as plant-derived MITEs are concerned, however, no such transposase-encoding open reading frame has been discovered. Therefore, many points remain unelucidated concerning the mechanisms of transposition of plant MITEs and concerning their actions.

By the way, the gene transfer experiments so far made in gene manipulation in higher living organisms are mostly of the nuclear genome insertion type. This is because higher living organisms have no equivalent to the plasmid in prokaryotes.

For such gen transfer by insertion into the nuclear genome, there are available physical methods

comprising introducing a vector coupled with a desired gene for insertion into the nuclear genome of a higher living organism by the particle gun, lipofection or electroporation technique, and biological methods comprising introducing said vectors harboring the genes once into a virus or microorganism and then introducing the same into the nuclear genome of a higher living organism by taking advantage of the DNA transfer/insertion system which said virus or microorganism has.

These physical and biological methods, however, each has a problem in that the expression activity of the gene inserted in a higher living organism varies from an individual to another, hence is not constant. In many instances, this is caused by silencing due to the position effect (Peach et al., Plant Mol. Biol. 17: 46-60 (1991)). Such the position effect is a phenomenon found in yeasts and many other eukaryotes and it is known that while there is no change in the nucleotide sequence of the inserted gene itself, the expression activity thereof varies markedly depending on the site of insertion of said gene on the chromosome and, in certain cases, the expression of the gene is completely inactivated (gene silencing) (Molecular Biology of the C ll, 3rd

edition, 434-435 (1995)).

Supposed as the causes of such phenomenon of the gene silencing are the fact that all genes in the nuclear genome of a higher living organism are not uniformly transcribed and the fact that active sites where the gene is actively transcribed and the cryptic sites where transcription of the gene is silenced at all are intermingled in the nuclear genome. It is known that a gene inserted in a cryptic site cannot be transcribed at all due to complete failure of proteins necessary for transcription to approach the gene or due to a change in nucleosome structure or heterochromatinization as resulting from methylation of the genomic DNA in this site, with the result that inactivation of gene expression (gene silencing) occurs (Ng et al., Curr. Opin. Genet. Dev., 9: 158-163 (1999); Matzke et al., Curr. Opin. Plant Biol. 1: 142-148 (1999)).

It is further known that, even in the same organism, this gene silencing depends on the state of cell differentiation, as seen with X chromosome of manmal. Furthermore, it is known that once gene silencing is caused by a presently unknown mechanism, it is inherited by the offspring of the next g neration resulting from mating (M lecular Biology

of the Cell, 3rd edition, 434-453 (1995)).

Meanwhile, when, in gene manipulation, a desired gene (foreign gene) is introduced into higher animal cells by a physical or biological method (other than the homologous recombination method used for gene knockout), the site of insertion of the gene in the cell nuclear genome differs among cells and it is substantially impossible to control it artificially. Thus, introduction of a foreign gene into cells of a higher living organism results in formation, in an uncertain manner, of cells containing the foreign gene in an active site of the nuclear genome and cells containing the gene in a cryptic site of the genome.

In the case of plant, it is known that a foreign gene, once introduced in a cryptic site, undergoes the influence of the cryptic site, with the result that the expression of the foreign gene markedly diminishes or becomes null and it is also known that even when a foreign gene is inserted in an active site, the expression of the foreign gene becomes unstabilized and diminishes due to gene silencing as the growth progresses by repetitions of cell division (Peach et al., Plant Mol. Biol. 17: 46-60 (1991)).

A main presumabl caus of inactivation of the expressi n of a for ign gene is the structure of the

nuclear DNA in the chromosome. An element participating in the structure of the nuclear DNA is a MAR (matrix attachment region; also called SAR (scaffold attachment region) sequence. This was found as a sequence anchoring a nuclear genomic DNA to a nuclear matrix. In animals, it was shown that when a MAR-containing chicken A element is ligated to a foreign gene to be inserted followed by gene transfer, the expression of the gene inserted increased (Stief et al., Nature 341; 343-345 (1989)). Later studies, however, revealed that the MAR contributes to an increase in gene expression efficiency but it, when alone, cannot counteract the position effect (Bonifer et al., Nculeic Acids Res. 22: 4202-4210 (1994); Poljak et al., Nucleic Acids Res. 22: 4386-4394 (1994)). In plants as well, studies have been made to investigate the effects of MARs using transformants. However, any reproducible results have not been obtained as yet. It is not considered that a MAR alone can counteract the position effect (Meshi: Shokubutsu no Genome Science (Genome Science of Plants), 153-160 (1996), published by Shujunsha).

Currently, genetically modified plants have been developed as genetically modified foodstuffs.

However, the most important problem to b solved in developing them is the phenomenon of gene silencing which causes inactivation of the expression of foreign genes, as mentioned hereinabove. In developing genetically modified plants, it is considered necessary, for avoiding the phenomenon of gene silencing, to select, among a very large number of plant individuals, plant individuals having a foreign gene inserted at a site where gene silencing is caused as scarcely as possible and, further, to select plant individuals in which gene silencing will not occur even after numerous generation for many years. For such selection, it is necessary to raise a large number of plant individuals and repeat over a number of generations and, therefore, not only much labor and time are required but also a vast area of land is required, namely a soil area problem arises.

Therefore, as another strategy of avoiding gene silencing, it is an urgent and most important problem in genetic engineering of plants to develop a method of suppressing the position effect-due gene silencing or activating the transcription of an inserted foreign gene and, more specifically, develop the so-called "transcriptional activation elem nt" having either f th se ffects.

DISCLOSURE OF INVENTION

As mentioned hereinabove, many points remain unelucidated as to what functions plant MITEs have. As for the functions of MITEs, the possibility is presumable of their contributing to the activation of gene expression based on the finding obtained by studies so far made that MITEs are found frequently in regions upstream of promoters of various genes (Wessler et al., Curr. Opin. Genet. Dev. 5: 914-821 (1995)). Further, since a number of MITEs are found in the vicinity of a matric attachment region (MAR) (Avramova et al., Nucleic Acids Res. 26: 761-767 (1998)), it is also presumable that they have an important connection with the structure of the genome.

Thus, MITEs are very interesting transposable elements (insertion elements) from the scientific viewpoint in elucidating the relation between the genomic structure and the gene expression, which has not yet been exhaustively investigated. In view of the possibility of MITEs, when incorporated in a genomic structure after transposition between genomic genes, changing the genomic structure and, as a result, controlling the gene expr ssion, their

utility as factors stabilizing, or preventing inactivation of, the gene expression activity on the occasion of foreign gene introduction can also be expected.

Thus, in a first aspect, the present invention has for its object to provide novel, plant-derived MITE-like elements, which have scientific and industrial utility, as mentioned above.

Specifically, the invention relates, first of all, to the novel MITE-like elements mentioned below under 1 to 5 (hereinafter, such MITE-like elements are sometimes referred to also as "IS2 elements" for convenience):

1. A MITE-like element capable of causing duplication of the target sequence: (A)nG(A)n [n being an integer of not less than 1];

2. A MITE-like element as defined above under 1 which has a perfect or imperfect terminal inverted repeat sequence in each of the 5' and 3' terminal regions;

3. A MITE-like element as defined above under 1 or 2 which contains a plurality of repetitions of at least one of the nucleotide sequences represented by the formula (1): XttgcaaY (wherein X represents g or t and Y represents a or c) or the formula (2): Zatgcaa (wh rein Z represents t r a);

4. A MITE-like element as defined above under any of 1 to 3 which has, as terminal inverted repeat sequences, a nucleotide sequence shown under SEQ ID NO:1 in the 5' terminal region and a nucleotide sequence shown under SEQ ID NO:2 in the 3' terminal region; and

5. A MITE-like element comprising the nucleotide sequence shown under SEQ ID NO:3.

The present invention further relates to the novel MITE-like elements mentioned below under 6 and 7 (hereinafter, such MITE-like elements are sometimes referred to also as "IS1 elements" for convenience):

6. A MITE-like element which has, as terminal inverted repeat sequences, a nucleotide sequence shown under SEQ ID NO:4 in the 5' terminal region and a nucleotide sequence shown under SEQ ID NO:5 in the 3' terminal region, and is capable of causing duplication of the target sequence TA; and

7. A MITE-like element comprising the nucleotide sequence shown under SEQ ID NO:6.

In its second aspect, the present invention has for its object to provide the so-called "transcriptional activation factor" capable of suppressing the gen inactivation phenomenon called gene silencing due t a position effect or capable

of activating the transcription of a gene located in the vicinity or marginal region thereof.

Specifically, the invention relates to the following transcriptional activation factors mentioned below under 8 to 12:

8. A transcriptional activation factor containing at least one transposable element;

9. A transcriptional activation factor as defined above under 8, in which the transposable element is a MITE-like element;

10. A transcriptional activation element as defined above under 9, in which the transposable element comprises a MITE-like element comprising the following DNA (a) or (b):

(a) a DNA having the nucleotide sequence shown under SEQ ID NO:1;

(b) a DNA capable of hybridizing with a DNA having the above nucleotide sequence (a) under stringent conditions and coding for a MITE-like element capable of causing duplication of (A)nG(A)n [n being an integer of not less than 1] at the site of insertion thereof, and/or a MITE-like element comprising the following DNA (c) or (d):

(c) a DNA having th nucleotide sequenc shown under SEQ ID NO:2;

(d) a DNA capable of hybridizing with a DNA having the above nucleotide sequence (c) under stringent conditions and coding for a MITE-like element capable of causing duplication of TA at the site of insertion thereof;

11. A transcriptional activation element as defined above under 9, in which the transposable element is a tandem coupling product from a MITE-like element comprising the following DNA (a) or (b):

(a) a DNA having the nucleotide sequence shown under SEQ ID NO:1;

(b) a DNA capable of hybridizing with a DNA having the above nucleotide sequence (a) under stringent conditions and coding for a MITE-like element capable of causing duplication of (A)nG(A)n [n being an integer of not less than 1] at the site of insertion thereof, and a MITE-like element comprising the following DNA (c) or (d):

(c) a DNA having the nucleotide sequence shown under SEQ ID NO:2;

(d) a DNA capable of hybridizing with a DNA having the above nucleotide sequence (c) under stringent conditions and coding for a MITE-like element capable of causing duplication of TA at the site of insertion th reof.

12. A transcriptional activation element comprising a DNA having the nucleotide sequence shown under SEQ ID NO:3.

The present invention further relates to a cassette for expression of a gene introduced which comprises the transcriptional activation element mentioned above. Specifically, there may be mentioned the cassettes mentioned below under 13 to 15:

13. A cassette for expression, in a plant, of a gene introduced which comprises the transcriptional activation element defined above under any of 8 to 12, and a DNA sequence operatively joined to said factor;

14. An introduced gene expression cassette as defined above under 13, in which the DNA sequence operatively joined to the transcriptional activation element comprises a promoter and/or a terminator;

15. An introduced gene expression cassette as defined above under 14 which further comprises, as the DNA sequence operatively joined to the transcriptional activation element, a desired introduced gene sequence to be expressed.

The present invention further r lat s to a plasmid which contains th transcriptional

activation element mentioned hereinabove (as an introduced gene expression cassette, for instance) and to a transgenic plant harboring the transcriptional activation element introduced therein by utilizing such plasmid.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a representation of the structure of an IS2 element, which is a MITE-like element according to the invention.

Fig. 2 is a representation of the structure of an IS1 element, which is a MITE-like element according to the invention, and of its terminal inverted repeat sequences and its inserted duplicate sequence (TA in the underlined parts).

Fig. 3 is a schematic representation of a method of constructing gDCPAL3-pro/SK.

Fig. 4 is a comparative representation of the structures of the carrot PAL genes gDCPAL3 and gDCPAL4.

Fig. 5 is a representation of the results of comparison between the nucleotide sequence of a MITE-like element (IS1 element) of the present invention and the nucleotide sequences of the so far known *Stowaway* family (Bureau and Wessler, Plant Cell,

6: 907-917 (1994). Sequences indicated by white letters on a black background are terminal inverted repeat sequences showing homology.

Fig. 6 is a representation of the imperfect inverted repeat sequences and target duplication sequence (AAAAGAAAA in the underlined parts) terminally found in an IS2 element, which is a MITE-like element of the invention.

Fig. 7 is a schematic representation of a method of constructing IS1-35S/SK.

Fig. 8 is a schematic representation of a method of constructing IS2-35S/SK.

Fig. 9 is a schematic representation of a method of constructing IS12-35S/SK.

Fig. 10 is a schematic representation of a method of constructing MU3-35S/SK.

Fig. 11 is a schematic representation of a method of constructing pIS1-35S/AB35S, pIS2-35S/AB35S, pIS12-35S/AB35S and pMU3-35S/AB35S.

Fig. 12 is a representation of the results of Example 3 (1) in which a comparison was made among the numbers of regenerated calli, on selection media containing kanamycin, from cultured tobacco BY-2 cells transform d by introduction of the constructs pIS1-35S/AB35S (IS1), pIS2-35S/AB35S (IS2),

pIS12-35S/AB35S (IS12) and pAB35S (35S) (control). In the figure, the upper and lower graphs show the results obtained by using selection media containing 100 μg/ml and 300 μg/ml of kanamycin, respectively.

Fig. 13 is a representation of the results of Example 3 (2) in which a comparison was made between the GUS activity of tobacco calli (control) resulting from introduction of pAB35S (35S) (left graph) and the GUS activity of tobacco calli resulting from introduction of pIS12-35S/AB35S (IS12) (right graph).

BEST MODES FOR CARRYING OUT THE INVENTION

I. Novel MITE-like elements

The known MITESs so far discovered include not only the *Tourist* and *Stowaway* families mentioned above but also the families *Castaway*, *Crackle*, *Emigrant*, *Explorer*, *Ditto*, *Gaijin*, *Krispie*, *Pop*, *Snap*, *Wanderer*, *Wujin*, *Wukong*, *Wuneng* and *Xbr*. While these MITEs are structurally characterized by all having a perfect or imperfect invereted repeat sequence in the terminal region, the inverted repeat sequences quite differ in nucleotide sequence and length among the MITEs. Furth r, the target duplication sequence in the MITE is TAA for *Tourist*, TA for *Stowaway*, TAA

for *Castaway*, GTTGATAT for *Crackle*, TTA for *Ditto*, TA for *Emigrant*, ATT or TAG or TGA or GGT or GTT or GAA for *Gaijin*, TTGAAC for *Krispie*, AAAACAAA or AAAAAAAA for *Pop*, TTTTTTT for *Snap*, TTA or TAA for *Wanderer*, TA or CATA for *Wujin*, TATA or TACA for *Wukong*, TTAA or TTAT for *Wuneng*, and TTAA for *Xbr* (any definite target duplication sequence has not been found for *Explorer*) (Bureau et al., Proc. Natl. Acad. Sci. USA, 93: 8524-8529 (1996); Casacuberta et al., Plant J., 16: 79-85 (1998); Song et al., Mol. Gen. Genet., 258: 449-456 (1998); Tu, Proc. Natl. Acad. Sci. USA, 94: 7475-7480 (1997); Unsal and Morgan, J. Mol. Biol., 248: 812-823 (1995)).

On the other hand, the IS2 element, an embodiment of the above-mentioned novel MITE-like element of the present invention has, as the target duplication sequence, (A)nG(A)n [n being an integer not less than 1], which is not found in any of the so far known MITEs (insertion elements). In this respect, the IS2 element can be said to be an insertion element belonging to a novel family different from any of the so far known families.

As for the IS1 element, which is an embodiment of the novel MITE-like element of the pr s nt invention, it has TA as the targ t duplication

sequence and thus can be said to be a novel MITE-like element belonging to the known family *Stowaway* (Bureau, T. E. and Wessler, S. R., *Stowaway*: a new family of inverted repeat elements associated with the genes of both monocotyledonous and dicotyledonous plants. Plant Cell, 6: 907-16 (1994)).

In the following, these IS2 and IS1 elements are described.

1. IS2 element

The IS2 element is characterized by causing target duplication of (A)nG(A)n in the genomic gene at the site of insertion. The number n may be any integer not less than 1. While it is not particularly restricted, it is specifically, for example 2 to 6, preferably 3 to 5, more preferably 4.

More specifically, the MITE-like element of the present invention is a DNA having a size of not more than about 2 kb, preferably about 0.2 to 2 kb, has repeat sequences reverse in direction to each other (terminal inverted repeat sequences) in the 5' and 3' terminal regions thereof.

From such viewpoint, the IS2 element of the present invention meets the requirements concerning the above-mention d three characteristics of MITEs (Wessler et al. Curr. Opin. Genet. Dev. 5: 914-821

(1995)), namely (1) that they hav a perfect or imperfect inverted repeat sequence at each of the 5' and 3' termini, (2) that a repeat sequence comprising two or more base pairs is found as a target duplicate sequence in the same direction on both sides of the inverted repeat sequence of the gene insertion site and (3) that their size is not more than 2 kb, hence can be identified as a transposable element (insertion element, MITE-like element) having a MITE-like sequence.

The IS2 element of the present invention is structurally characterized by containing, in the nucleotide sequence thereof, at least one nucleotide sequence represented by the formula (1): XttgcaaY (wherein X represents g or t and Y represents a or c) (SEQ ID NO: 7 to 10) or the formula (2): Zatgcaa (wherein Z represents t or a) (SEQ ID NO: 11 to 12) in a continuously or discontinuously repeated manner.

The positioning and number of such repeat sequences are not particularly restricted but they may be contained in the terminal inverted repeat sequences occurring in both the terminal regions of the IS2 element or in an intermediate region occurring b twe n said terminal inverted repeated s quences.

The IS2 element of the present inventi n

specifically includes the ones which contain a plurality of repeat sequences represented by the above formula (1) and (2) in the intermediate region between the terminal inverted repeat sequences and a plurality of repeat sequences represented by the formula (1) in the terminal inverted repeat sequences, as shown in Fig. 1.

The terminal inverted repeat sequences which the IS2 element of the present invention has need not be strictly complementary to each other but the only requirement is that the 5' and 3' terminal regions can hybridize with each other under stringent conditions and, as a result, the IS2 element can have such a stem structure as shown in Fig. 1. In this sense, the IS2 element of the present invention includes not only those having perfect inverted repeat sequences as the terminal inverted repeat sequences but also those having imperfect inverted repeat sequences as the terminal inverted repeat sequences.

As specific examples of the IS2 element according to the present invention, there may be mentioned the ones which have, as the terminal inverted r peat sequences, the nucleotide sequence shown under SEQ ID NO:1 in the 5' terminal r gion and

the nucl otide sequence shown under SEQ ID NO:2 in the 3' terminal region. As a more specific example of the IS2 element, there may be mentioned the one having the nucleotide sequence shown under SEQ ID NO:3. The IS2 element may have one or more nucleotides substituted, added or deleted in the terminal inverted repeat sequences or in the sequence occurring between said repeat sequences if the resulting modifications remain functional equivalents substantially having the function or activity of the IS2 element itself. The MITE-like element of the present invention includes such functional equivalents as well.

As preferred functional equivalents, there may be mentioned the ones substantially having the function or activity of the IS2 element having the nucleotide sequence shown under SEQ ID NO:3, and causing target duplication of (A)nG(A)n [n being an integer not less than 1] at the site of insertion and capable of hybridizing with the above IS2 element under stringent conditions. As "stringent conditions", there may be mentioned the conditions in 1 × SSC plus 0.1% (w/w) SDS at 50°C or above over a period of 1 hour. As th functional equivalents, there may be mentioned more specifically th ones n t

less than 70%, preferably not less than 85%, more preferably not less than 90%, still more preferably not less than 95% homologous in nucleotide sequence with the IS2 element shown under SEQ ID NO:3.

2. IS1 element

The IS1 element of the present invention brings about target duplication of TA at the site of genomic gene insertion and is characterized by having, as the terminal inverted repeat sequences, the nucleotide sequence shown under SEQ ID NO:4 in the 5' terminal region and the nucleotide sequence shown under SEQ ID NO:5 in the 3' terminal region. The IS1 element of the invention is specifically a DNA having a size of not more than about 1 kb, preferably about 100 bp to 500 bp. In the light of such facts, the IS1 element of the invention can be defined as a MITE-like element, like the IS2 element mentioned above.

As the IS1 element of the present invention, there may specifically be mentioned the one having the structure shown in Fig. 2. More specifically, there may be mentioned the one having the nucleotide sequence shown under SEQ ID NO:6. The MITE-like element having such nucleotide sequence may have one or more nucl otid s substituted, add d or d leted in the terminal inverted repeat s quences or in the

sequence occurring between these repeat sequences of the 5' and 3' terminal regions if the resulting modifications remain functional equivalents substantially having the function or activity of the MITE-like element itself. The MITE-like element of the present invention includes such functional equivalents as well.

Preferred as the functional equivalents are those which substantially have the function or activity of the MITE-like element (IS1 element) having the nucleotide sequence shown under SEQ ID NO:6 and which are at least 85%, preferably at least 90%, more preferably at least 95% homologous in nucleotide sequence with said IS1 element.

The MITE-like elements (IS2 and IS1 elements) described hereinabove all have been discovered from the carrot genome, more specifically from the carrot phenylalanine ammonia-lyase gene, as mentioned later herein, and can be isolated and recovered as described later herein in the example section. The MITE-like element of the present invention is not limited in origin provided that it has the structure and characteristics mentioned hereinabove.

It is generally pointed out that transp sable elements, as s lf-mechanisms of automodification of

the plant genome itself, might possibly contribute markedly to evolution of the organism concerned and to environmental adaptation.

As regards the MITE-like element of the present invention, it is unknown as to the mechanisms by which it functions in relation to the self-mechanisms of automodification of plant genomes. However, unlike the case of retrotransposons (McDonald, BioScience 40: 183-191 (1990), such a fact that it has an enhancer element therewithin and a gene promoter is activated by insertion of said enhancer element as resulting from transposition of said element is not found.

Therefore, the MITE-like element of the present invention can be considered to highly possibly cause changes in genomic structure upon insertion thereof in a plant genome and thereby contribute to dynamic changes of the genomic structure, such as changes in unwindability of the genomic DNA or in nucleosome structure, by the mechanisms quite different from those of the so far known enhancer elements.

With the MITE-like element of the present invention, it becomes possible, by utilizing the above property, to control the expression of a gene located in the vicinity of said element by a technique different from the so far known techniques. It is

generally pointed out that when a foreign gene is inserted in a cryptic site of a genomic DNA, the expression thereof is suppressed or inactivated. Therefore, it is considered possible, by utilizing the MITE-like element of the present invention and based on the above properties, to invigorate or activate the reduced or suppressed ability to be expressed of a foreign gene introduced by transgenic technique. Accordingly, the MITE-like element of the present invention is useful in constructing a transgene expression cassette and a plasmid containing said cassette in stably creating genetically engineered plants and also useful in stably creating genetically engineered organisms capable of expression of the transgene by utilizing said cassette and plasmid.

II. Transcriptional activation element

The present invention further relates to a transcriptional activation element.

The transcriptional activation element so referred to herein includes a element capable of promoting the transcription of a group of genes located in the vicinity or marginal region of said element as w ll as a element capable of inhibiting suppression of a desired foreign gene introduced in

a genomic DNA or the like from being inactivated by gene silencing due to the position effect. The so-far known elements (factors) in charge of transcriptional activation each activates the transcription of a specific gene by occurring, as an enhancer, in the vicinity of the promoter of said gene and cis-acting directly on said promoter. On the contrary, the transcriptional activation element of the present invention promotes the transcription of a single gene or a group of a plurality of genes located in the vicinity or marginal region thereof, irrespective of position relative to the promoter and, further, includes those substantially promoting the transcription by suppressing the inherent phenomenon of transcription inactivation, hence conceptually includes a broader range of factors as compared with the prior art concept of transcriptional activation element.

The transcriptional activation element of the present invention is characterized by containing at least one transposable element.

The transposable element so referred to herein includes all of the above-mentioned DNA type and RNA type ones and MITEs. Thus, th transcriptional activation element of the present invention includes

those containing at least one of these as a transposable element, regardless of whether it is derived from the same species or a different species.

A preferred transcriptional activation element contains a MITE(s) as the transposable element.

While a MITE is defined, as mentioned above, as an element having such characteristics as (1) having a perfect or imperfect inverted repeat sequence in each of both 5' and 3' terminal regions (similar in this respect to DNA type transposable elements), (2) having, on both sides of the inverted repeat sequence, a target duplication sequence comprising repeat sequences arranged in the same direction and comprising two or more base pairs, like the ones formed upon insertion of a DNA type transposable element in a genomic DNA and (3) having a size generally of shorter than 2 kb (Wessler et al., Curr. Opin. Genet. Dev. 5: 914-821 (1995)), any MITE belonging to such category of definition can be used as the transposable element in carrying out the present invention. As MITEs particularly suited among others, there may be mentioned the above-mentioned novel MITE-like elements of the present inv ntion, namely the "IS2 lement", "IS1 lement", and functional equivalents thereto.

Thus, the transcriptional activation element of the present invention preferably contains at least one nucleotide sequence selected from among said IS2 element, IS1 element, and functional equivalents thereto.

More specifically, the transcriptional activation element of the present invention includes, among others, (1) the one having the nucleotide sequence of the IS1 element or a functional equivalent thereof, (2) the one having the nucleotide sequence of the IS2 element or a functional equivalent thereof, (3) the one having a nucleotide sequence resulting from tandem joining of the nucleotide sequence of the IS1 element or a functional equivalent thereof and the nucleotide sequence of the IS2 element or a functional equivalent thereof (the order of the IS1 element and IS2 element being arbitrary) and (3) the one having a nucleotide resulting from joining of the nucleotide sequence of the IS1 element (or IS2 element) or a functional equivalent thereto and the nucleotide sequence of the IS2 element (or IS1 element) or a functional equivalent thereto via an arbitrary nucleotide sequence. Preferred examples of the transcriptional activation factor, th re may be mentioned (i) the IS2 element or a functional

equivalent thereto and (ii) the product of tandem joining of the IS1 element (or IS2 element) or a functional equivalent thereto and the IS2 element (or IS1 element) or a functional equivalent thereto. As a specific example of the latter (ii), there may be mentioned the one having the nucleotide sequence shown under SEQ ID NO:3.

Referring to the transcriptional activation element mentioned above under (4), the intervening nucleotide sequence between the IS1 element and IS2 element (the order being arbitrary) is not particularly restricted but may be any nucleotide sequence on condition that the effects of the invention are not counteracted. As a specific, but nonlimitative, example, there may be mentioned one derived from the carrot PAL promoter sequence. Generally, such nucleotide sequence can have a size of 5 to 1,000 bp, preferably 300 to 500 bp. As a transcriptional activation element of such mode of embodiment, there may be specifically mentioned the one having the nucleotide sequence shown under SEQ ID NO:14.

The transcriptional activation element of the present invention can be operatively joined to a desired gene sequence to be introduced into a plant

body (transgene sequence (inclusive of foreign gene sequence)) and, further, the transcriptional activation element joined to said transgene sequence can be operatively joined to a functional DNA sequence or sequences, such as a promoter functional in a plant and/or a terminator functional in a plant.

The expression "operatively joined" as used herein means that the transcriptional activation element is located at a position sufficiently close to the above-mentioned transgene sequence or various functional DNA sequences to exert its influence on these sequences, irrespective of insertion site and direction.

The transgene to be used in the practice of the present invention includes those DNAs which are desired to be expressed in plants, whether homologous or heterologous to said plants. Such transgene includes, but is not limited to, genes coding for β-glucuronidase; antibiotic resistance genes; genes coding for insecticidal and bactericidal proteinaceous toxins; genes for antipathogenic compounds; genes synthesizing hypersensitivity compounds, such as peroxidases, glucanases, chitinases, and phytoalexins; agroch mical, herbicid and micr bicide resistance genes; genes

synthesizing plant enzymes (e.g. enzymes connected with the contents and qualities of proteins, starch, saccharides and fats) and genes for regulatory factors therefor; genes related to plant enzyme inhibitors, such as protease and amylase inhibitors; genes involved in plant hormone synthesis; genes involved in insect hormone and pheromone synthesis; genes involved in the synthesis of medicinal and nutritional compounds, such as β-carotene and vitamins; and antisense transcripts interfering with nucleotide sequences occurring in plants (Transgenic Plant, vol. 1, Academic Press, 1993).

The present invention further relates to a gene expression cassette suited for application to plants, which comprises the above transcriptional activation element and a DNA sequence or sequences operatively joined thereto. The "gene expression cassette" so referred to herein means a plasmid to be used for introduction into plants as well as a subfragment thereof.

As the DNA sequence operatively joined to the transcriptional activation element, there may be mentioned functional DNA sequences such as promoter and terminator. Said DNA sequence can include any of the above-mentioned transgene sequences.

The promoter, so referred to herein, means a DNA sequence which, when the structural gene for a desired protein is joined thereto downstream from said promoter, can regulate the expression of said protein via transcription followed by translation in plant cells, and it includes all promoters functional in plants and used in the relevant field of art for the transformation of plants. A number of promoters have so far been used in transforming plants, including, for example, the promoters isolated from Agrobacterium tumefaciens, namely the octopine synthase (ocs) promoter (L. Comai et al., 1985; C. Waldron et al., 1985), mannopine synthase (mas) promoter and nopaline synthase (nos) promoter. The cauliflower mosaic virus 35S promoter, which is generally used in the transformation, can adequately be used in practicing the present invention. Modifications of the 35S promoter, for example the two parallel 35S promoters (R. Kay et al., 1987) and the mas-35S promoters (L. Comai et al., 1990), can also be used. Furthermore, the cauliflower mosaic virus 19S promoter (J. Paszkowski et al., 1984) and scrophularia mosaic virus-derived 34S promoter (M. Sanger et al., 1990) can also be included. As further examples, there may be mentioned the actin promoter,

ribulose-1,5-bisphosphate carboxylase small subunit (rbcS) promoter and so forth, which are plant-derived promoters.

The terminator includes DNA sequences capable of efficiently terminating the transcription of a desired structural gene in plants and includes all terminators functioning in plants that are used in the relevant field of art for the transformation of plants. Specifically, the nopaline synthase (nos) terminator, for example, can be mentioned as a typical example.

The transcriptional activation element of the present invention or the transgene expression cassette comprising said element can be used for inducing or regulating the expression in a plant of the gene introduced, and this widely applies to plants in general.

The plant is not particularly restricted but includes, among others, agriculturally useful plants, whether monocotyledonous or dicotyledonous. Among the monocotyledonous plants, for instance, there are corn, rice, wheat, barley, African or Indian millet, oat, rye, millet and other cereal crop plants as well as lily, orchid, iris, palm, tulip, sedge and oth r various ornamental plants. The dicotyledons include

chrysanthemum, snapdragon, carnation, magnolia, poppy, cabbage, rose, pea, poinsettia, cotton, cactus, carrot, cowberry, peppermint, sunflower, tomato, elm, oak, maple tree, poplar, soybean, melon, beet, rape, potato, lettuce, carica papaya, etc.

The present invention further provides a transgenic plant which contains the transcriptional activation element of the present invention or the transgene expression cassette of the present invention comprising said element and in which the phenomenon of the position effect-due gene silencing (inactivation) has been suppressed for the desired foreign gene introduced or the transcription of the foreign gene has been activated. Said transgenic plant includes the offspring thereof.

The term "plant" as used herein is intended to include not only a perfect plant body but also a portion of a plant body, such as a leaf, seed, bulb or cutting. It further includes protoplasts, plant calli, mericlones and like plant cells as well.

The method of producing such transgenic plant is not particularly restricted but may be any of those DNA introduction methods which are conventionally used in the relevant field of art. Sp cifically, it is a method of introducing a DNA into plant cells using

an expression plasmid containing the transcriptional activation element of the present invention or a transgene expression cassette containing said element and includes, among others, such known methods as the Agrobacterium method, electric method (electroporation) and particle gun method.

The thus-obtained plant cells containing the transcriptional activation element of the invention, the transgene expression cassette containing said element, or the expression plasmid can be regenerated by one of the conventional methods used in the plant tissue culture technology as described, for example, in S. B. Gelvin, R. A. Schilperoot and D. P. S. Verma: Plant Molecular Biology Manual (1991), Kluwer Academic Publishers or Valvekens et al., Proc. Natl. Acad. Sci., 85: 5536-5540 (1988), whereby plant bodies, or portions thereof, derived from said plant cells can be obtained.

The expression plasmid of the present invention may be any one provided that it contains the desired DNA sequence to be introduced (transgene) together with the transcriptional activation element and functional DNA sequences such as a promoter and a terminator. It is preferred, however, that th se DNA sequences be op ratively joined to one another. The

phrase "operatively joined" means that the plasmid functions for the intended purpose. Specifically, it is implied that when the plasmid in question is introduced into plant cells, the desired transgene (structural gene) is expressed under the control of the transcriptional activation element and the expression is efficiently terminated by the action of the terminator, without inactivation of the promoter contained in said plasmid.

The present invention further includes a method of causing expression of a transgene in a plant. Such method can be carried out at least by the step of introducing, into a plant, the transgene expression cassette with the transgene integrated therein, such as mentioned above, and the step of causing expression of said transgene in said plant. The introduction of the transgene expression cassette (DNA) into the plant and the expression of said transgene can both be effected by the techniques per se known in the art (Plant Molecular Biology Manual, 1991, Kluwer Academic Publishers).

EXAMPLES

The following examples illustrate the present invention in further detail. They are, however, by

no means limitative of the scope of the present invention. The genetic engineering techniques and the experimental procedures of molecular biology (restriction enzyme treatment conditions, ligation reaction conditions, transformation into *Escherichia coli*, etc.), which are to be employed in the practice of the present invention, can be carried out as generally and widely employed, for example as described in J. Sambrook, E. F. Frisch, T. Maniatis: Molecular Cloning, 2nd edition, Cold Spring Harbor Laboratory Press, 1989 and D. M. Glover: DNA Cloning, IRL Press, 1985, among others.

Example 1

1) Target plant, target gene

In searching for MITE-like elements, carrot (*Daucus carota* L. cv. Kurodagosun) was used as the target plant, and the phenylalanine ammonia-lyase (PAL) of said carrot as the target gene.

2) Cloning of the carrot PAL genes gDCPAL3 and gDCPAL4 Carrot genomic sequences were cloned from a carrot genomic DNA library. The carrot genomic DNA library was constructed, as previously described by the present inventors (Ozeki, Y., Davies, E. and Takeda, J.: Structure and expression of chalcone synthase gene in carrot susp nsi n cultured c lls

regulated by 2,4-D. Plant Cell Physiol., 34: 1029-1037 (1993)), from cultured carrot cells (Ozaki, Y. and Komamine, A.: Induction of anthocyanin synthesis in relation to embryogenesis in a carrot suspension culture; Correlation of metabolic differentiation with morphological differentiation. Physiol. Plantarum, 53: 570-577 (1981)) using the λ EMBL3 vector (product of Toyobo).

Specifically, carrot genomic DNA was prepared from carrot freeze-dried using a cetyltrimethylammonium bromide (CTAB) solution according to the method of Murray and Thompson (1980) (Murray, M. G. and Thompson, W. F.; Rapid isolation of high molecular weight plant DNA. Nucl. Acids Res. 8: 4321-4325 (1980)). The genomic DNA obtained was partially digested with *Sau*3AI and the digested DNA was fractionated by size by the sucrose density gradient method. The DNA fraction within 15 to 20 kbp was collected and ligated to the *Bam*HI-digested λ EMBL3 vector, followed by packaging in phage particles, to thereby construct a carrot nuclear library.

Then, the carrot genomic library was screened (Sambrook et al., 1989) for carrot PAL genomic clones using th PAL cDNA (ANT-PAL cDNA) as th probe cloned

by the method of Ozeki et al. (Ozeki, Y., Matsui, K., Sakuta, M., Matsuoka, M., Ohashi, Y., Kano-Murakami, Y., Yamamoto, N, and Tanaka, Y.: Differential regulation of phenylalanine ammonia-lyase genes during anthocyanin synthesis and by transfer effect in carrot cell suspension cultures. Physiol. Plantarum, 80: 379-387 (1990)). The hybridization for screening of the carrot genomic library was effected by overnight treatment at 68°C with a solution containing 6 × SSC, 60 mM sodium phosphate (pH 6.8), 10 mM EDTA, 1% SDS, 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400 and 100 μg/ml denatured salmon sperm DNA. The membrane washing was carried out twice (15 minutes × 2) in a 2 × SSC solution containing 0.5% SDS at room temperature, twice (10 minutes × 2) in a 0.1 × SSC solution or a 1 × SSC solution containing 0.1% SDS at room temperature and, finally, twice (30 minutes × 2) in a 0.1 × SSC solution at 68°C.

As a result, eight positive clones were obtained. Restriction enzyme maps were prepared for such clones and, according to the maps, the clones were classifiable into two genes, which were named *gDCPAL3* and *gDCPAL4*, respectiv ly.

With *gDCPAL3*, the λ phage of a positive clon

was cultured, λ DNA was extracted, cleaved with *Bam*HI and subjected to Southern transfer to a nylon membrane, according to the methods described in Sambrook et al. (1989). With this, Southern analysis was carried out using a probe prepared from the above-mentioned ANT-PAL cDNA by cleavage with *Eco*RI and labeling a DNA fragment (984 bp) thereof at the 5' end with [^{32}P], and a 2.77 kbp DNA fragment hybridizing with said probe was obtained. This DNA fragment was cleaved with *Bam*HI and subcloned in the pBluescript SK plasmid treated with calf intestine alkaline phosphatase (CIP), to give gDCPAL3-pro/SK (cf. Fig. 3). Then, the plasmid gDCPAL3-pro/SK obtained was cleaved with the restriction enzymes *Sal*I and *Apa*I, a series of deletion DNA fragment groups were produced using exonuclease III and mung bean nuclease by the method described by Sambrook et al. (1989), and the nucleotide sequence of the *gDCPAL3* DNA was determined using them. The site of transcription initiation (+1) was determined based on the positions of bands as found by the primer extension method using mRNA extracted from carrot as described by Ozeki and Takeda (1994) (Regulation of phenylalanine ammonia-lyase genes in carrot suspension cultured cells. Plant Cell. Tissue and Organ Culture, 38: 221-225 (1994)).

With *gDCPAL4*, the plasmid gDCPAL4-pro/SK was produced and the nucleotide sequence of the *gDCPAL4* DNA corresponding to the above was determined in the same manner. Specifically, the λ DNA obtained from the λ phage of a positive *gDCPAL4* clone was cleaved with *Hind*III and *Bam*HI, Southern analysis was performed using the same probe as mentioned above, and a 1.63 kbp DNA fragment hybridizing with the probe was cleaved with *Hind*III and *Bam*HI and subcloned in the pBluescript SK plasmid treated with CIP, to give gDCPAL4-pro/SK. Then, the thus-obtained plasmid gDCPAL4-pro/SK was cleaved with the restriction enzymes *Xba*I and *Bst*XI, a series of deletion DNA fragment groups were produced using exonuclease III and mung bean nuclease by the method described by Sambrook et al. (1989), and the nucleotide sequence of the *gDCPAL4* DNA was determined using them.

3) Results

Comparison of the nucleotide sequences of *gDCPAL3* and *gDCPAL4* revealed that the promoter region of *gDCPAL3* has miniature inverted-repeat transposable elements (MITEs) having imperfect inverted repeat sequences not found in *gDCPAL4* were present at two sites, namely
-1897 to -1599 (299 bp in length) and -1157 to -389

(769 bp in length) (Fig. 4). These sequences were named IS1 and IS2, respectively.

These sequences and the nucleotide sequences around the sites of insertion were sequenced using an autosequencer (product of LICOR model 4000L). The nucleotide sequence of IS1 is shown under SEQ ID NO:2 and the nucleotide sequence of IS1 under SEQ ID NO:1.

The characteristics of these IS1 and IS2 were as follows:

(1) IS1

It had the nucleotide sequence shown under SEQ ID NO:2 (total length: 299 bp), had inverted repeat sequences (32 bp), which were imperfect to each other, in the 5' and 3' terminal regions, and had a target duplication sequence, TA, at the site of insertion into the genome serving as the target gene. Based on these facts, it was estimated to be the gene sequence of a novel MITE element belonging to the family *Stowaway* already reported (Bureau, T. E. and Wessler, S. R. *Stowaway*: a new family of inverted repeat elements associated with the genes of both monocotyledonous and dicotyledonous plants. Plant Cell, 6: 907-16 (1994)). The stem structure of the element IS1 and th structure of th t rminal inverted r p at sequ nce region and of the ins rti n site

region are shown in Fig. 2.

Based on the nucleotide sequence information obtained, homology analysis of the nucleotide sequence was performed using commercial databases (e.g. GENE TYX-MAC/CD1995), whereupon, in the terminal inverted repeat sequences, 70-90% homology was found with the gene sequence of MITE elements belonging to the *Stowaway* family (Bureau and Wessler (1994)). It was thus confirmed that said element is a transposable element belonging to the *Stowaway* family (Fig. 5).

(2) IS2

It had the nucleotide sequence shown under SEQ ID NO:1 (total length: 769 bp), had inverted repeat sequences (158 bp), which were imperfect to each other, in the 5' and 3' terminal regions, and had a target duplication sequence, AAAAGAAAA, at the site of insertion into the genome serving as the target gene. Homology comparison of the nucleotide sequence was made but no homology with known transposable elements was detected. It was thus found that it is a transposable element, particularly a MITE-like element, constituting a novel family belonging to none of the so far known transposable element families. The verall structure (stem structure) of the IS2

element is shown in Fig. 1 and the structure (nucleotide sequence) of its terminal inverted repeat sequence region and of the insertion site region in Fig. 6.

Example 2

(1) Cloning of IS1, IS2, IS12 and MU3

(i) Cloning of IS1 (Fig. 7)

From among the plasmids having a deletion DNA fragment derived from the 3' terminus of the *gDCPAL3* promoter region as prepared for nucleotide sequence determination in Example 1, a plasmid (gDCPAL3-IS1/SK) with deletion to -1581 was selected, and a 321 bp DNA fragment was excised by cleaving that plasmid with *Kpn*I, rendering blunt-ended using T4 DNA polymerase and cleaving with *Sca*I, followed by agarose gel electrophoresis. This was subcloned in the plasmid pBluescript SK cleaved with *Hinc*II and treated with CIP. Plasmids were extracted from among *Escherichia coli* colonies harboring a plurality of independent clones obtained by the above subcloning and the nucleotide sequences thereof were determined for revealing the direction of each DNA fragment inserted. In this way, a plasmid with the 5' terminal side of IS1 being ins rted on th *Kpn*I side of the multiple cloning site of the pBluescript SK plasmid

was selected and named IS1/SK. Further, the cauliflower mosaic virus 35S promoter (35S) fragment obtained by cleavage of pBI221 (Clontech Inc.) with *Hind*III and *Sma*I was recovered by agarose gel electrophoresis and subcloned in the pBluescript SK plasmid cleaved with *Hind*III and *Sma*I and treated with CIP, to give a plasmid named 35S/SK. From IS1/SK, the insert DNA fragment was excised by cleaving with *Kpn*I and *Hind*III, followed by agarose gel electrophoresis, and this was subcloned in the 35S/SK plasmid cleaved with *Kpn*I and *Hind*III and treated with CIP, to give IS1-35S/SK having the IS1 region in the upstream region of the 35S promoter region.

(ii) Cloning of IS2 (Fig. 8)

From among the plasmids having a deletion DNA fragment derived from the 3' terminus of the *gDCPAL3* promoter region as prepared for nucleotide sequence determination in Example 1, a plasmid (gDCPAL3-IS12/SK) showing deletion to -389 was selected, and a 797 bp DNA fragment was excised by cleaving that plasmid with *Kpn*I, rendering blunt-ended using T4 DNA polymerase and cleaving with *Dde*I, followed by agarose gel electrophoresis. This was subcloned in the plasmid pBluescript SK cl aved with *Hinc*II and tr ated with CIP. Plasmids were extracted from among

E. coli colonies harboring a plurality of independent clones obtained by the above subcloning and the nucleotide sequences thereof were determined for revealing the direction of each DNA fragment inserted. In this way, a plasmid with the 5' terminal side of IS2 being inserted on the *Kpn*I side of the multiple cloning site of the pBluescript SK plasmid was selected and named IS2/SK-1. One with the 3' terminal side of IS2 being inserted on the *Kpn*I side, namely in the reverse direction, was selected and named IS2/SK-2 (reverse).

IS2/SK-1 was cleaved with *Kpn*I and *Hind*III and the insert DNA fragment was recovered by agarose gel electrophoresis, and this was subcloned in the 35S/SK plasmid cleaved with *Kpn*I and *Hind*III and treated with CIP, to give IS2-35S/SK having the IS2 region (positive strand) in the upstream region of the 35S promoter region.

(iii) Cloning of IS12 (IS1-IS2 tandem coupling product) (Fig. 9)

The IS2/SK-2 (reverse) obtained as described above under (ii) was cleaved with *Kpn*I, rendered blunt-ended using T4 DNA polymerase and then cleaved with *Hind*III, and the insert DNA fragment was recov red by agarose gel lectrophoresis. This was

cleaved with *Pst*I, rendered blunt-ended using T4 DNA polymerase and then subcloned in the IS1-35S/SK plasmid cleaved with *Hind*III and treated with CIP, to give IS12-35S/SK having the IS1 region and IS2 region in the upstream region of the 35S promoter region in a tandem manner.

(iv) Cloning of MU3 (Fig. 10)

As for MU3, gDCPAL3-IS12/SK was cleaved with *Kpn*I, then rendered blunt-ended using T4 DNA polymerase and cleaved with *Sca*I, and a 1,514 bp DNA fragment was recovered by agarose gel electrophoresis. This was subcloned in the pBluescript SK plasmid cleaved with *Hinc*II and treated with CIP. Plasmids were extracted from a plurality of *E. coli* colonies harboring each independent clone as obtained by the above subcloning and the nucleotide sequences thereof were determined to thereby check the direction of DNA fragment insertion. A plasmid with the 5' terminal side of IS1 inserted on the *Kpn*I side of the multicloning site of the pBluescript SK plasmid was selected and named MU3/SK. The insert DNA fragment was excised by cleaving MU3/SK with *Kpn*I and *Hind*III, followed by agarose gel electrophoresis. This was subcloned in the 35S/SK plasmid cleaved with *Kpn*I and *Hind*III and treat d with CIP, t give MU3-35S/SK

having the IS1 region and IS2 region, via a *gDCPAL3*-derived region sequence (441 bp), in the upstream region of the 35S promoter region.

(2) Insertion of IS1, IS2, IS12 and MU3 into a plant gene expression vector (Fig. 11)

pABN-Hm1 (Mita, S., Suzuki-Fujii, K. and Nakamura, K. Sugar-inducible expression of a gene for β-amylase in Arabidopsis thaliana. Plant Physiol., 107: 895-904 (1995); gift from Dr. Kenzo Nakamura at Nagoya Univeristy) was cleaved with *Hind*III to thereby excise the β-amylase promoter (1.7 kb), which was rendered blunt-ended using T4 DNA polymerase, then cleaved with *Xba*I, treated with CIP and then subjected to agarose gel electrophoresis, whereby a 10 kbp DNA fragment containing the Ti plasmid region as well as the kanamycin resistance gene [nos promoter/coding region of neomycin phosphotransferase II gene (nptII)/nos terminator], the coding region (GUS)/nos terminator of the β-glucuronidase gene, and the hygromycin resistance gene [35S promoter/coding region (HPT) of hygromycin phosphotransferase gene/nos terminator] was isolated. In this was subcloned a 35S fragment obtained from 35S/SK by cleavage with *Hind*III, rend ring blunt-ended using T4 DNA polymerase and cleaving with *Xba*I,

to construct pAB35S.

This was cleaved with *Xho*I and *Xba*I and treated with CIP and then a vector was prepared by cutting off the 35S DNA fragment by agarose gel electrophoresis. Separately, the IS1-35S/SK, IS2-35S/SK, IS12-35S/SK and MU3-35S/SK prepared as mentioned above were each cleaved with *Xho*I and *Xba*I and DNA fragments for insertion (transgene expression cassettes) were recovered by agarose gel electrophoresis. Such DNA fragments were ligated to the vector mentioned above and used to transform *E. coli* DH5α. LB agar medium containing 25 μg/L of kanamycin (1% Bacto-Trypton, 0.5% yeast extract, 1% sodium chloride, 1.5% agar powder for bacterial culture media) was sowed with each of the *E. coli* transformants obtained, and plasmids were extracted from the colonies obtained by the rapid plasmid DNA extraction method and the restriction enzyme maps of the plasmids obtained were checked, whereby the formation of the constructs pIS1-35S/AB35S, pIS2-35S/AB35S, pIS12-35S/AB35S and pMU3-35S/AB35S, namely the constructs with the above transgene expression cassettes respectively inserted between the nptII gen r sponsible for kanamycin resistance and the GUS gene, which is a structural gene, was

confirmed as shown in Fig. 11.

(3) Production of competent cells of *Agrobacterium tumefaciens*

A YEP solid medium (prepared by adding powdered agar for bacterial culture media to YEP medium comprising 1% yeast extract, 1% Bactoheptone and 0.5% sodium chloride to a concentration of 1.5%, followed by solidification by autoclaving; hereinafter the same shall apply) was smeared with a loopful of cells taken from a glycerol stock of *A. tumefaciens* EHA 101 and the cells were cultured at 28°C in the dark for 2 days. Grown single colonies of *A. tumefaciens* were each collected with a toothpick and sowed in 1.5 ml of YEP medium and shake-cultured overnight at 28°C. 80 ml of YEP medium was placed in a 500-ml flask, 0.8 ml of the *A. tumefaciens* culture fluid was added, and shake culture was performed at 28°C until OD_{600} = 0.4. This was cooled with ice, transferred to a centrifuge tube ice-cooled in advance, and centrifuged at 6,000 rpm at 4°C for 5 minutes, the supernatant was removed, and 20 ml of 10% glycerol was added to suspend the sediment. This procedure was repeated three times, and the medium was completely removed to give comp tent cells of *A. tumefaciens*. For stocking, the cells were suspended in 400 μl of 10% glyc rol and

the suspension was distributed in 40-μl portions into tubes, followed by rapid freezing in liquefied nitrogen.

(4) Introduction of plasmid DNAs into A. *tumefaciens*

The constructs obtained as described above under (2) (plasmids pIS1-35S/AB35S, pIS2-35S/AB35S, pIS12-35S/AB35S and pMU3-35S/AB35S) were each introduced into the competent cells of A. *tumefaciens* by electoporation (using Shimadzu GTE-10). Specifically, about 100 ng each of the plasmids prepared as described above under (2), namely pIS1-35S/AB35S (IS1), pIS2-35S/AB35S (IS2), pIS12-35S/AB35S (IS12) and pMU3-35S/AB35S (MU3), and the plasmid pAB35S (35S) to serve as a control with no insertion of the tanscriptional activation element(s) (IS1 and/or IS2) was admixed with 40 μl of competent cells prepared as described above under (3), and each mixture was transferred to an electroporation cell. Electric pulses (1.2 kV, 35 μF, 550 Ω) were given, and 1 ml of YEP medium was immediately added, and incubation was performed at 28°C for 1 hour. About 50 μl was taken and YEP solid medium containing 50 μg/L of hygromycin was smeared th rewith, and incubation was performed in the dark at 28°C for 2 days. A monocol ny that had grown was

again spread lightly over another portion of YEP solid medium containing 50 μg/L of hygromycin using a platinum loop and incubated at 28°C for 24 hours. A portion of cells were taken and planted in 5 ml of YEP medium containing 50 μg/L of hygromycin and shake-cultured overnight at 28°C.

Example 3

(1) Introduction of transcriptional activation element-containing constructs into tobacco cultured cells

Using A. tumefaciens with the construct prepared in Example 2, namely pIS1-35S/AB35S (IS1), pIS2-35S/AB35S (IS2) or pIS12-35S/AB35S (IS12), introduced therein (hereinafter referred to as "transformant *A. tumefaciens*"), the constructs IS1, IS2 and IS12 were respectively introduced into tobacco cultured cells. In a control run, *A. tumefaciens* with pAB35S (35S) introduced therein was used and the same procedure was followed.

First, the above transformant *A. tumefaciens* cultured in 5 ml of YEP liquid medium was transferred to a 50-ml centrifuge tube and centrifuged at 3,000 rpm for 10 minutes. The supernatant was discarded, 25 ml of Linsmaier & Skoog medium (Linsmaier, E. M. and Skoog, F.; Physiol. Plantarum 18, 100-127 (1965);

hereinafter referred to as "Lins medium") was added to the sediment and, after resuspending, centrifugation was carried out again at 3,000 rpm and at room temperature for 10 minutes, and the supernatant was discarded. This procedure was repeated four times. Cells of *A. tumefaciens* were harvested, Lins medium was added for suspending in an amount to give OD_{600} = 0.2, acetosyringone was added thereto to a concentration of 10 μg/ml, followed by resuspending.

Saparately, cultured tobacco cells BY-2 (gift from Dr. Toshiyuki Nagata at University of Tokyo) to be used for introduction of each construct were cultured beforehand in 45 ml of Lins medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), 1 ml of the cell-containing suspension culture fluid was transferred to a fresh portion of Lins medium at one-week intervals, and cells that had entered the logarithmic growth phase after the lapse of about 100 hours following transfer were used.

A sterile 90-mm dish was sowed with said tobacco cultured cells BY-2 (4 ml), and 100 μl of cells of transformant *A. tumefaciens* washed by the above procedure were added uniformly onto the tobacco cultured cells. After slight blending, co-culture

was carried out in the dark at 22°C for 3 days.

Then, 12 ml of Lins medium was added to the cultured cell fluid for suspending, the suspension was transferred to a 50-ml centrifuge tube and centrifuged at 1,000 rpm for 1 minute, and the supernatant was discarded. This procedure was repeated four times. Then, 12 ml of Lins medium containing 250 μg/ml of claforan was added and the same procedure as mentioned above was once more repeated. After discarding the supernatant, about 25 ml of Lins medium was added to the sediment cells to thereby suspend them, and the cells in 0.25 μl of the medium were counted using a hemocytometer. The cells were uniformly sowed onto portions of Lins solid selection medium containing 100 μg/ml or 300 μg/ml of kanamycin (further containing 250 μg/ml of claforan) so that the number of cells per plate amounted to 4×10^5, 6×10^5, 8×10^5, 10×10^5 or 15×10^5. They were cultured in the dark at 28°C. After one month of culture, the number of transgenic cultured tobacco cells (transformant calli) formed on each plate and the formation rate or yield were determined. The results thus obtained are shown in Table 1 and Fig. 12.

Table 1

Number of transformant calli formed from cultured tobacco cells BY-2 in kanamycin-containing medium and rate of transformant callus formation

Kanamycin 100 μg/ml

Number of cells (× 10^5)	4	6	8	10
IS1	4±0	20±4	43±3	99±10
	(0.10)	(0.33)	(0.54)	(0.99)
IS2	10±7	72±13	84±4	137±12
	(0.25)	(1.20)	(1.05)	(1.37)
IS12	16±3	70±6	124±16	220±4
	(0.40)	(1.67)	(1.55)	(2.20)
35S (Control)	3±2	40±8	47±2	83±13
	(0.08)	(0.67)	(0.59)	(0.83)

Kanamycin 300 μg/ml

Number of cells (× 10^5)	4	6	8	10
IS1	0	3±0	10±3	20±5
	(0.00)	(0.05)	(0.13)	(0.20)
IS2	0	4±2	36±4	72±11
	(0.00)	(0.07)	(0.45)	(0.72)
IS12	2±1	15±1	41±2	89±5
	(0.05)	(0.25)	(0.51)	(0.89)
35S (Control)	0	0	3±2	37±16
	(0.00)	(0.00)	(0.04)	(0.37)

Upper: Number of calli formed

Lower: Yield (× 10^{-2} %)

From the above results, it was found that

insertion of the construct IS2 or IS12 into tobacco cultured cells increases the yield of transformant calli in kanamycin-containing medium and that the yield is higher than the yield of transformant calluses from cultured tobacco cells (35S) without insertion of such element. Specifically, the yield of transformant calli (transgenic cultured tobacco cells) with the construct IS2 or IS12 introduced therein was 1.6 to 2.6 times higher as compared with the control (35S) without introduction of such element.

Particularly when the kanamycin concentration in medium was 300 μg/ml, it was observed that, by introducing the construct IS2 or IS12, the yield of transformant calli is increased to a level 10 times or more higher as compared with the control (35S). This suggests that insertion of the construct IS2 or IS12 result in an increased level of expression of the ntpII gene responsible for kanamycin resistance and occurring in the vicinity or marginal region of said construct.

(2) GUS activity measurement in transformant tobacco calli

Based on the abov results, the expression of the GUS gen , which is a r porter, was checked using

the above pIS12-35S/AB35S (IS12) as the construct, to thereby check whether the above inserted construct can increase the expression of the structural gene.

The expression of the GUS gene was examined by first randomly selecting independent calli from among a plurality of transformant tobacco calli, extracting nuclear DNA from each callus and, after confirming the gene introduction by Southern analysis, extracting proteins from the callus and measuring the GUS activity. Specifically, 0.75 g of the transformed tobacco callus was taken and proteins were extracted using GUS-Light (Tropix, Inc.). The protein concentration was determined using Bio-Rad's protein assay kit and then, using GUS-Light (Tropix) and the luminometer Lumat LB905 (Bertold Japan), the GUS activity was measured for 5 seconds. The results thus obtained are shown in Fig. 13. In the figure, the GUS activity (ordinate) is shown in terms of luminescence value per unit weight of protein as calculated by dividing the luminescence value obtained from the luminometer by the protein weight.

As is evident from Fig. 13, the transformant tobacco calli resulting from insertion of the construct IS12 showed, on an average, about 2.6 tim s higher GUS activity as compared with the control

resulting from insertion of element-free pAB35S (35S). From this, it was found that the expression of the GUS gene is significantly increased by insertion of the construct IS12.

This indicates that each element (IS1 element, IS2 element, and a coupling product therefrom (e.g. IS12 element)) contained in the above constructs is a transcriptional activation element.

Example 4 Introduction of a transcriptional activation element-containing construct into a tobacco plant (leaf disk method)

Using *A. tumefaciens* with the construct prepared in Example 2, namely pIS1-35S/AB35S (IS1), pIS2-35S/AB35S (IS2) or pIS12-35S/AB35S (IS12), introduced therein (hereinafter referred to as "transformant *A. tumefaciens*"), the constructs IS1, IS2 and IS12 were respectively introduced into tobacco leaves by the leaf disk method. In a control run, *A. tumefaciens* with the construct-free pAB35S (35S) introduced therein was used and the same procedure was followed.

Specifically, each tobacco (SR 1) leaf was immersed in a 10% hypochlorous acid solution, air bubbles were removed using a medicine spoon over 2 minutes with gentle stirring by means of a stirrer,

the solution was renewed and the same procedure was repeated for further 5 minutes. The leaf was taken out and immersed in sterilized water, followed by gentle stirring. While replacing the hypochlorous acid solution with a fresh portion, the same procedure was repeated three times in all. After removing the moisture using a sterilized paper towel, the leaf was punched with a cork borer to give a leaf disk (the vein being removed). This was immersed in 10 ml of sterilized water. The thus-prepared leaf disk was immersed in the above-mentioned transformant *A. tumefaciens* cultured in 5 ml of YEP medium (adjusted to OD_{600} = 0.25 with sterilized water). Then, the bacterial suspension and the leaf disk were together emptied onto a sterilized paper towel and the moisture was removed with another sterilized paper towel.

The leaf was placed, inside out, on MS infection medium prepared by supplementing MS medium (Murashige, T. and Skoog, F.; Physiol. Plantarum 15, 473-497 (1962)) with 40 mg/L acetosyringone and 0.2% gelan gum, and cultured in the dark at 25° C for 2 days. Then, each leaf disk was deprived of bacterial cells in the manner of wiping with MS differentiation medium (MS m dium supplemented with 0.1 mg/L α-naphthaleneacetic acid, 1 mg/L benzyladenine, 150

mg/L kanamycin, 500 mg/L claforan and 0.2% gelan gum), then placed, inside out, on another portion of MS differentiation medium and cultured at 25°C. The medium was replaced with a fresh portion of MS differentiation medium at two-week intervals and, after the lapse of one month, regenerated shoots were counted. The results thus obtained are shown in Table 2.

Table 2
Comparison in number of regenerated shoots on tobacco leaf disks

Number of shoots per disk	35S	IS1	IS2	IS12
0	73	49	56	50
1	26	21	20	23
2	13	17	11	14
3	10	14	8	5
4	3	6	9	8
5	2	1	2	1
6	1	3	3	2
7	0	1	2	5
8	1	0	0	3
9	0	0	0	1
10	0	0	2	1
11	0	0	0	1
12	0	0	0	1
13	0	0	0	0
14	0	0	0	2
Total number of disks	129	112	113	117
Total number of shoots	118	151	164	244
Average number of shoots per disk	0.91	1.35	1.45	2.09

As is evident from the above results, the shoot regeneration efficiency was about 1.4 times when the tobacco plant contained the construct IS1 or IS2 as compared with the element-free control (35S) and, in particular when it contained both IS1 and IS2 in a tandem manner (IS12), about twice as many shoots were obtained as compared with the control. From this, it is evident that the elements of the present invention (IS1 element, IS2 element and coupling products obtained therefrom (IS12 and the like)) can increase the activity of the kanamycin gene (ntpII gene) occurring in the vicinity or marginal region of said elements in the tobacco plant. This result supports the judgment drawn in Example 3 that the elements of the present invention are transcriptional activation elements.

Example 5 Introduction of transcriptional activation element-containing constructs into carrot somatic embryos

Using *A. tumefaciens* with the construct prepared in Example 2, namely pIS1-35S/AB35S (IS1), pIS2-35S/AB35S (IS2), pIS12-35S/AB35S (IS12) or pMU3-35S/AB35S (MU3), introduced therein (hereinafter referred to as "transformant *A. tumefaciens*"), the constructs IS1, IS2, IS12 and MU3 were respectively

introduced into carrot somatic embryos. In a control run, *A. tumefaciens* with the construct-free pAB35S (35S) introduced therein was used and the same procedure was followed.

Specifically, first, carrot hypocotyls germinated under the sterilized condition were cut to a length of about 1 cm, then placed in MS medium containing 4.5×10^{-6} M 2,4-D (2,4-dichlorophenoxyacetic acid) and cultured in the dark for 24 hours, then placed in 2,4-D-free MS medium and cultured in the dark for 3 days. The medium was replaced with a fresh portion and cultivation was performed in the same manner for 7 days to initiate carrot somatic embryos on the hypocotyls.

Separately, a culture of the above transformant *A. tumefaciens* cultured in 5 ml of YEP medium was centrifuged at 3,000 rpm for 10 minutes, the supernatant was removed, and about 30 ml of MS medium was added to suspend the cells. This procedure was repeated twice and the YEP medium was completely removed. Then, centrifugation was carried out at 3,000 rpm for 10 minutes, the supernatant was removed, and MS medium containing 10 mg/L of acetosyringone was added to the sedim nt to th r by adjust to OD_{600} = ab ut 0.3.

To this were added the above carrot hypocotyls collected using a net, and the mixture was shaken gently for 5 minutes. The hypocotyls were deprived of the moisture by wiping with a sterilized paper towel and immersed in MS medium containing 10 mg/L of acetosyringone and cultured in the dark at 22°C for 3 days. The hypocotyls were deprived of the moisture by wiping with a sterilized paper towel and immersed in MS medium containing 500 μg/L of carbenicillin and washed with the medium by shaking gently. After removing the moisture in the same manner, the hypocotyls were cultured in MS agar medium (containing 0.8% agar) containing 500 μg/L of carbenicillin and 100 μg/L of kanamycin in the dark. After 1.5 to 3 months, hypocotyls that had each regenerated a callus were counted. The results thus obtained are shown in Table 3.

Table 3

Comparison in number of callus-regenerating hypocotyls

		35S	IS1	IS2	IS12	MU3
+ 2,4-D	Number of callus-regenerating hypocotyls	5	6	10	9	8
	Total number of hypocotyls	78	56	60	61	63
	Percent regeneration(%)	6.4	11	17	15	13
- 2,4-D	Number of callus-regenerating hypocotyls	3	9	12	6	4
	Total number of hypocotyls	77	62	62	64	58
	Percent regeneration (%)	3.9	13	19	9.4	6.9

As is evident from Table 3, with carrot somatic embryos as well, like the case of tobacco calli, the regeneration efficiency was found improved upon insertion of the construct IS1, IS2, IS12 and MU3 under the dedifferentiational growth conditions of culturing in 2,4-D-containing medium (+ 2,4-D) as well as under the differentiational growth conditions of culturing in 2,4-D-free medium (- 2,4-D). The greatest improvement in regeneration efficiency was observed in the case of the construct IS2 inserted.

Example 6 Introduction of transcriptional activation element-containing constructs into rice

Using *A. tumefaciens* with the construct prepared in Example 2, namely pIS1-35S/AB35S (IS1) or pIS2-35S/AB35S (IS2), introduced therein (hereinafter referred to as "transformant *A. tumefaciens*"), the constructs IS1 and IS2 were respectively introduced into rice seeds. In a control run, *A. tumefaciens* with the element-free pAB35S (35S) introduced therein was used and the same procedure was followed.

Specifically, from among fully ripened rice seeds (Nihonbare), those normal in shape and color, among others, wer first selected and dehulled by lightly rubbing in a mortar. Dehull d se ds were

placed in a 50-ml Falcon tube, 2.5% sodium hypochlorite was added, and the mixture was shaken at 100-120 rpm for 20 minutes. Then, the supernatant was discarded, sterilized water was added, and the mixture was shaken gently. After three repetitions of this procedure, the seeds were placed on callus induction medium and cultured in the dark at 28°C.

After 3 to 4 weeks, among calli formed from scutella and having growing yellowed shoots, only those having a diameter of 2-3 mm and looking like scattered in a group of several were placed on fresh callus induction medium and cultured in the dark at 28°C for 7 days.

Separately, the above transformant *A. tumefaciens* was planted in YEP solid medium and cultured in the dark at 28°C for 3 days. Cells of *A. tumefaciens* were scratched off with a medicine spoon and added to AAI medium (Toriyama, K. and Hirata, K.; Plant Science 41, 179-183 (1985)) supplemented with acetosyringone, and the OD_{600} was adjusted to 0.18 to 0.2. They were shake-cultured in the dark at 25°C for 1 hour.

The rice calli cultured in the above manner were placed in a sterilized tea strainer, and the above cultured *A. tumefaciens* in the form of a suspension

was added. The tea strainer was shaken for 3 minutes with occasional rocking for securing immersion of the whole calli, then the tea strainer and the contents were together placed on a paper towel, and the excessive bacterial culture fluid was removed. The calli were placed on co-culture medium and cultured in the dark at 25°C for 3 days.

The co-cultured calli were then collected in a tea strainer, immersed in sterilized water supplemented with 500 mg/l of claforan, the tea strainer was shaken to wash away *A. tumefaciens*, the tea strainer and the contents were then together placed on a sterilized paper towel, and the water was removed. The same procedure was repeated four times in all. The calli were placed on selection medium and cultured in the dark at 28°C. Three to four weeks later, calli were randomly selected from among a large number of calli, a portion of each selected callus was taken and placed in a GUS staining solution (0.75 mM X-Gulc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.3% Triton X-100, 20% methanol, 50 mM phosphate buffer (pH 7.0)), and the reaction was all wed to proceed ov rnight at 37°C for detecting GUS activity. The callus stained blue and

thus showing GUS activity is a transgenic, transformed rice callus. The results thus obtained are shown in Table 4.

Table 4

Results of rice callus staining for GUS

	35S	IS1	IS2
Calluses stained	27	38	44
Total number of calluses	275	244	228
Percentage of transformant calluses (%)	9.8	15.6	19.3

As is evident from the results shown in Table 4, the transformation efficiency was found increased by introduction of the constructs IS1 and IS2 to about 1.5 times and about 2 times, respectively, as compared with the control (35S).

The above-mentioned Examples 1 to 6 showed that the regeneration efficiency (transformation efficiency) is increased by using the transcriptional activation elements (IS1, IS2, IS12, MU3) of the present invention. The following three possibilities can be considered as the reasons:

(1) The possibility of the efficiency of introduction of Ti plasmid into plant cells being increased;

(2) The possibility of the fficiency of regeneration from c lls being incr ased as the result

of an increase in nos promoter activity owing to the IS1 and/or IS2 element and consequent promotion of the transcription of the nptII gene, which leads to production of the gene product in an increased amount, and, hence, increase in number of cells capable of growing on the kanamycin-containing medium used for selection; and

(3) The possibility of the IS1 and/or IS2 element activating the gene region in the vicinity or marginal region thereof or preventing said gene region from being inactivated.

The gene introduction by means of Ti plasmid does not lead to insertion at a determined site on the plant chromosome but is indefinite as to the site of insertion. Therefore, it is accidental whether the gene in question is inserted in an active site determined by the structure of the chromosome or in a cryptic site in the vicinity of which a gene has been inactivated by methylation of the genomic DNA or by some other cause. It is thought that if a transgene is introduced in a cryptic site, it is influenced by the "field" of the chromosome, so that the transgene is also inactivated.

In the constructs used in the above examples, the transcriptional activation elem nts (IS1 or/and

IS2) are found inserted on the terminator side of the nptII gene (kanamycin resistance gene), namely on the opposite side of the nos promoter of the kanamycin resistance gene. Therefore, it is impossible that these elements cis act on the promoter of the kanamycin resistance gene.

However, the above examples gave the results showing that even in the circumstances in which the transcriptional activation element of the present invention is found inserted in a position such that it cannot directly act on the nos promoter, the number of kanamycin resistant cells (transformant tobacco calli) increases in the case of cultured tobacco cells, in particular that even when the kanamycin concentration in medium is as high as 300 mM, the number of kanamycin resistant calli increases (Example 3 (1)), and further that the efficiency of regeneration of kanamycin resistant plants is increased by introducing the constructs mentioned above into plant cells of various plant species (Examples 4 to 6). These results indicate that the IS1 or/and IS2 elements acted on the kanamycin resistance gene occurring in the vicinity of said elements, not in th mode of directly causing cis activation of the nos prom ter, and as a r sult, the

number of cells retaining (includes both the senses of activation and prevention from being inactivated) the activity of said kanamycin resistance gene. Thus, this indicates the possibility that, unlike the conventional transcriptional activation elements (factors) occurring as enhancers in the vicinity of the promoter of a specific gene and activating the transcription by cis acting on said promoter, the transcriptional activation element of the present invention is to activate (or prevent from inactivating), when it is inserted into a genomic gene, a single gene group or a plurality of gene groups in the vicinity or marginal region of the site of insertion thereof (irrespective of location and direction of the gene promoter on which it acts) and thus promoting the transcription activity, namely acting by the mechanisms mentioned above under (3).

In the constructs used in the above examples, the IS1 or/and IS2 elements were inserted on the 35S promoter side of the GUS gene. The cultured tobacco cells resulting from insertion of said construct showed increased GUS activity in Example 3 (2) indicated clearly that the transcriptional activation elements of the inv ntion act also on the 35S promoter occurring in the downstream vicinity

thereof to increase the transcription activity of the GUS gene. This result supports the judgment mentioned above and indicates that the transcriptional activation elements of the invention show the actions mentioned above under (3), namely that "the IS1 or/and IS2 elements activate the neighboring gene region including them or prevent said gene region from being inactivated".

In the above example, it was also shown that, not only with cultured cells (Example 3) but also with plant tissues, the efficiency of regeneration of tobacco plant shoots is indeed increased in the tobacco leaf disk experiment (Example 4), the efficiency of formation of embryogenic callus serving as bases for plant regeneration from carrot hypocotyls is increased (Example 5), and the efficiency of formation of callus serving as bases for rice plant regeneration is increased (Example 6), by using the transcriptional activation elements of the invention. These results indicate that the transcriptional activation elements of the invention act on those plant cells becoming incapable of plant regeneration or callus formation as a result of a for ign gene introduced into the genome of plants in question undergoing gene silencing (inactivation) by

the position effect, so as to increase the efficiency of plant regeneration or formation, hence are practically very useful.

In view of the recent finding that MITEs, like MARs, bind to nuclear matrices (Tikhonov et al., Plant Cell 12: 249-264 (2000)), it is considered that MITEs intranuclearly play a role similar to that of MARs. Based on this, it can be expected that the transcriptional activation elements of the invention, which are MITEs, can be used singly or in combination with such elements as MARs in producing genetically modified plants to further increase the efficiency of plant regeneration or formation.

INDUSTRIAL APPLICABILITY

The most important problem to be overcome in producing genetically modified plants is the phenomenon of gene silencing which causes expression inactivation of foreign genes. For avoiding the gene silencing phenomenon in developing genetically modified plants, it is necessary to select, from among a large number of plant individuals, those plant individuals with the foreign gene in question inserted at such a site as causing gene silencing as least as possible.

The present invention provides novel, plant-derived MITE-like elements, and it is highly possible that said MITE-like elements, when inserted in a plant genome, cause changes in genomic structure and, based on this, contribute to changes in dynamics of the genomic structure, for example facilitating the unwinding of genomic DNA or causing changes in nucleosome structure, by mechanisms quite different from the mechanisms of action of the conventional enhancer elements.

Therefore, by utilizing this characteristic feature of the MITE-like elements of the invention, it will become possible to regulate the expression of a gene occurring in the vicinity thereof by techniques different from the prior art ones. In other words, with the MITE-like elements of the invention which have the above characteristic feature, it will be possible to increase or activate the reduced expression ability of a foreign gene introduced by the transgenic technology. In this respect, the MITE-like elements of the invention are useful in constructing a transgene expression cassette and plasmids containing said cassette in the production of genetically modifi d living organisms and is furth r useful in stably pr ducing genetically

modified organisms capable of expressing a transgene.

The transcriptional activation elements of the invention which contain a transposable element such as one of the MITE-like elements mentioned above (preferably the IS1 element or/and IS2 element) have an activity in suppressing and dissolving the phenomenon of inactivation of gene expression (gene silencing phenomenon) due to the position effect in gene transfer in plants. Therefore, it is expected that by using the transcriptional activation elements of the invention singly or in combination with other elements participating in nuclear DNA structuring, for example MARs (matrix attachment regions), it will become possible to have the gene stably expressed to thereby markedly reduce the number of screening procedures generally performed after gene transfer and the number of recombinant plants to be sowed and grown.

Such plants large in genomic size as lily, chrysanthemum and wheat have large cryptic sites within the genome and, therefore, a foreign gene introduced is mostly inserted in cryptic sites. For such plants, it is thus very difficult and practically impossible in the prior art to produc recombinant plants. On th c ntrary, with the transcriptional

activation elements of the invention, it is possible to significantly inhibit a foreign gene introduced onto a plant genome from undergoing silencing and therefore it is expected that said elements can make it possible to efficiently introduce foreign genes into those plant species the gene recombination of which has been regarded as difficult, as mentioned above, and produce recombinant plants.

The transcriptional activation elements of the invention are also considered to be elements capable of activating (inclusive of transcriptional activation) genes occurring in the vicinity or marginal region of the gene region in which they are found inserted. Therefore, the transcriptional activation elements of the invention are expected to not only make it possible to put the transgenic technology to practical use even in those plants in which the production of genetically modified plant bodies is difficult because of frequent occurrence of silencing due to the large genome size, as mentioned above, but also make it possible, even in the genetic engineering of plant species such as soybean, corn, potato and tomato, already put to practical use, to incr ase the efficiency of gene transfer and, at the same time, incr ase the activity

of transcription of genes for useful characters, such as herbicide resistance genes and insecticidal protein genes, by using these transcriptional activation elements through insertion at a site upstream of the promoter of such a gene for a useful character, or at a site in the vicinity thereof. They are further expected to make it possible to produce genetically modified plants with higher productivity and higher quality as compared with the conventional methods of producing genetically modified plants.

CLAIMS

1. A miniature inverted-repeat transposable element (MITE)-like element capable of causing duplication of the target sequence: (A)nG(A)n [n being an integer of not less than 1] at the site of insertion thereof in a genomic gene.

2. A MITE-like element as claimed in Claim 1 which has perfect or imperfect terminal inverted repeat sequences in the 5' and 3' terminal regions.

3. A MITE-like element as claimed in Claim 1 or 2 which contains, in the sequence thereof, a plurality of repetitions of at least one of the nucleotide sequences represented by the formula (1): XttgcaaY (wherein X represents g or t and Y represents a or c) or the formula (2): Zatgcaa (wherein Z represents t or a).

4. A MITE-like element as claimed in any of Claims 1 to 3 which has, as terminal inverted repeat sequences, a nucleotide sequence shown under SEQ ID NO:1 in the 5' terminal region and a nucleotide sequence shown under SEQ ID NO:2 in the 3' terminal region.

5. A MITE-like el ment comprising the nucleotide sequenc shown und r SEQ ID NO:3.

6. A MITE-like element which has, as terminal inverted repeat sequences, a nucleotide sequence shown under SEQ ID NO:4 in the 5' terminal region and a nucleotide sequence shown under SEQ ID NO:5 in the 3' terminal region, and is capable of causing duplication of the target sequence TA at the site of insertion thereof in a genomic gene.

7. A MITE-like element comprising the nucleotide sequence shown under SEQ ID NO:6.

8. A transcriptional activation element characterized by containing at least one transposable element.

9. A transcriptional activation element as claimed in Claim 8, wherein the transposable element is a MITE-like element.

10. A transcriptional activation element as claimed in Claim 9, wherein the transposable element comprises at least one MITE-like element comprising the following DNA (a) or (b):

(a) a DNA having the nucleotide sequence shown under SEQ ID NO:1;

(b) a DNA capable of hybridizing with a DNA having the above nucleotide sequence (a) under stringent conditions and coding for a MITE-like element capable of causing duplication of (A)nG(A)n [n being an

integer of not less than 1] at the site of insertion thereof in a genomic gene,

or a MITE-like element comprising the following DNA (c) or (d):

(c) a DNA having the nucleotide sequence shown under SEQ ID NO:2;

(d) a DNA capable of hybridizing with a DNA having the above nucleotide sequence (c) under stringent conditions and coding for a MITE-like element capable of causing duplication of TA at the site of insertion thereof in a genomic gene.

11. A transcriptional activation element as claimed in Claim 9, wherein the transposable element is a tandem coupling product from a MITE-like element comprising the following DNA (a) or (b):

(a) a DNA having the nucleotide sequence shown under SEQ ID NO:1;

(b) a DNA capable of hybridizing with a DNA having the above nucleotide sequence (a) under stringent conditions and coding for a MITE-like element capable of causing duplication of (A)nG(A)n [n being an integer of not less than 1] at the site of insertion thereof in a genomic gene,

and a MITE-like el ment comprising the following DNA (c) or (d):

(c) a DNA having the nucleotide sequence shown under SEQ ID NO:2;

(d) a DNA capable of hybridizing with a DNA having the above nucleotide sequence (c) under stringent conditions and coding for a MITE-like element capable of causing duplication of TA at the site of insertion thereof in a genomic gene.

12. A transcriptional activation element comprising a DNA having the nucleotide sequence shown under SEQ ID NO:3.

13. A transgene expression cassette which comprises the transcriptional activation element of any of Claims 8 to 12, and a DNA sequence operatively joined to said element.

14. A transgene expression cassette as claimed in Claim 13, wherein the DNA sequence operatively joined to the transcriptional activation element comprises a promoter and/or a terminator.

15. A transgene expression cassette as claimed in Claim 14, which further comprises, as the DNA sequence operatively joined to the transcriptional activation element, a desired transgene sequence to be expressed.

16. A plasmid containing the transcriptional activation element of any f Claims 8 to 12.

17. A plasmid containing the transgene expression cassette of any of Claims 13 to 15.

18. A transgenic plant which contains the transgene expression cassette of any of Claims 13 to 15.

19. A transgenic plant as claimed in Claim 18 which is corn, rice, wheat, lily, chrysanthemum, cotton, soybean, beet, potato or carica papaya.

FIG. 1

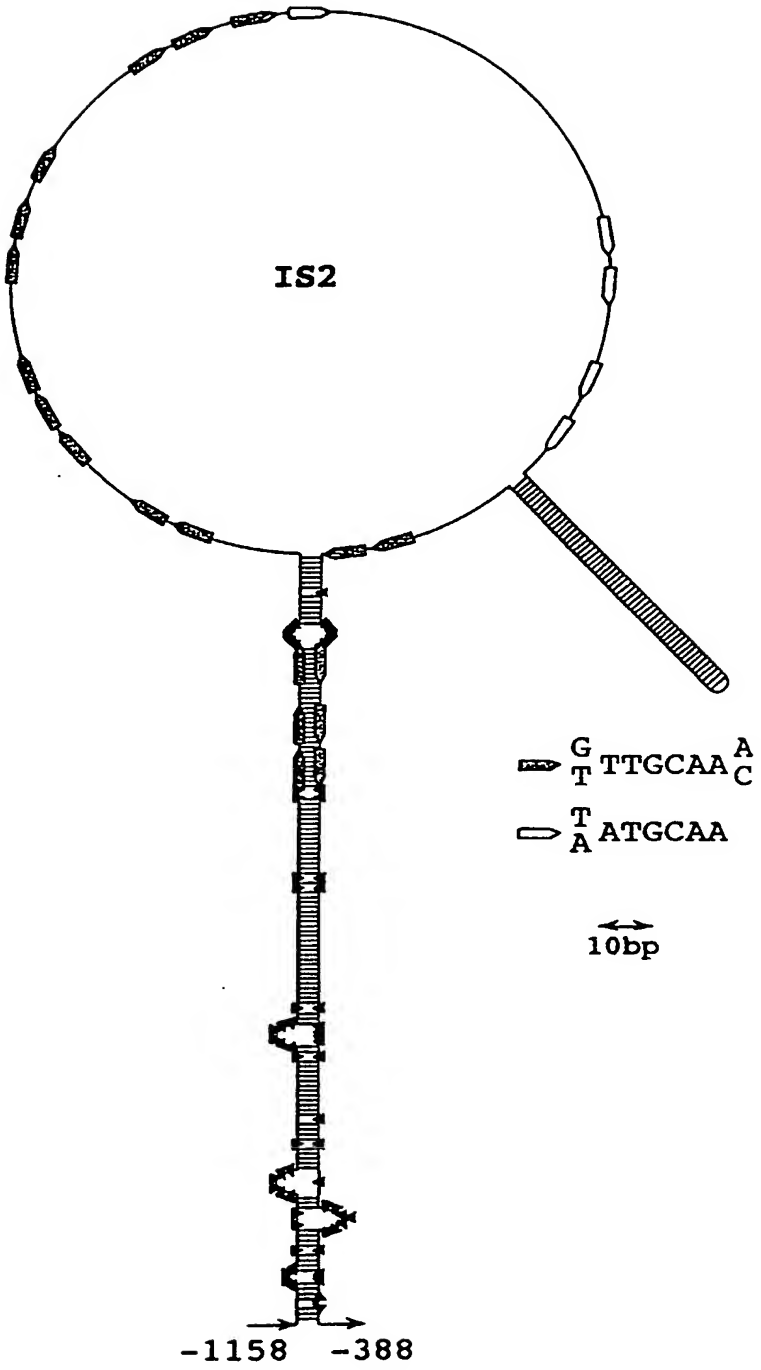


FIG. 2

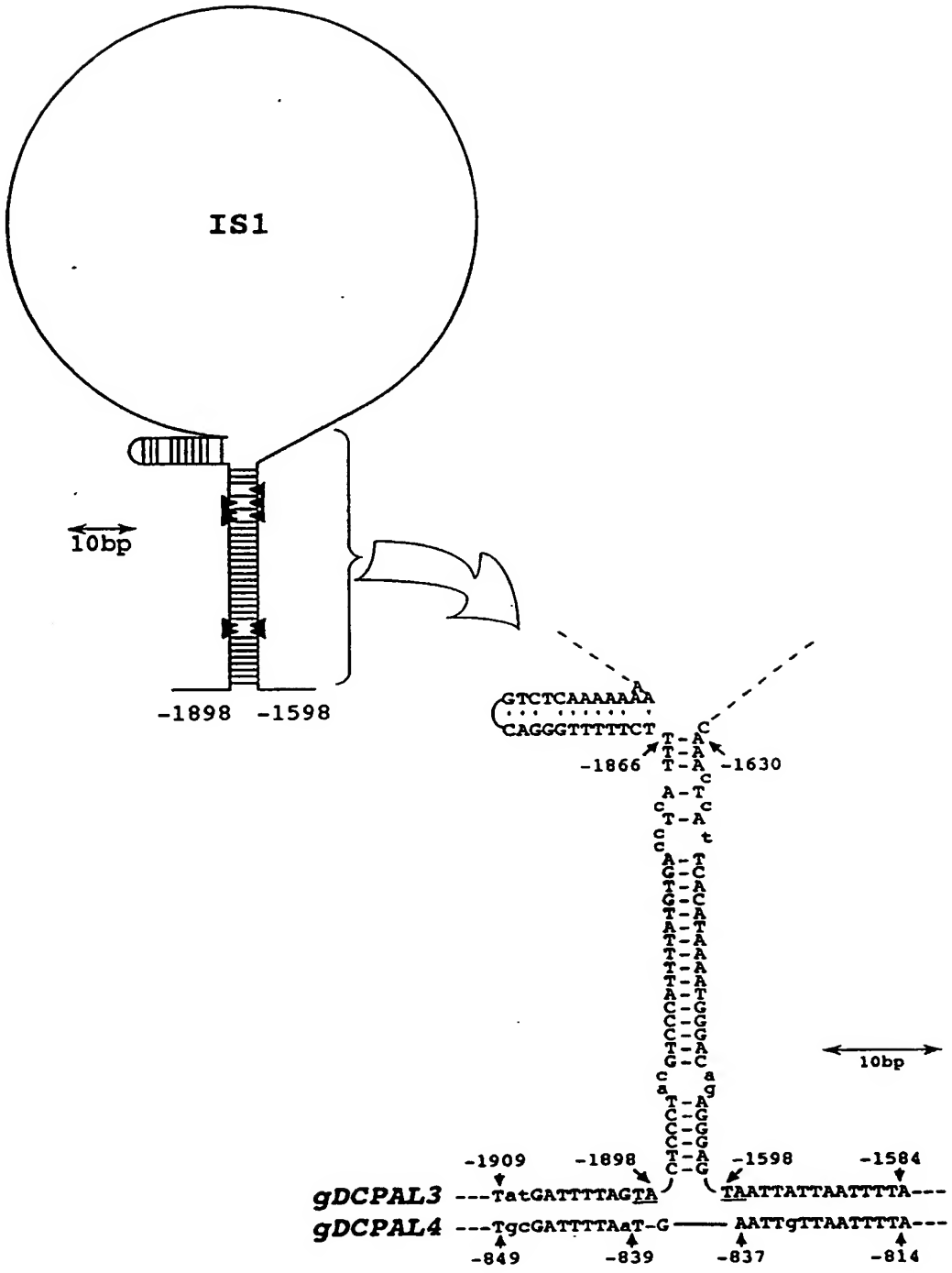

IS1
10bp
-1898
-1598
GTCTCAAAAAAA
CAGGGTTTTTCT
-1866
-1630
10bp
-1909
-1898
-1598
-1584
gDCPAL3 ---TatGATTTTAGTA TAATTATTAATTTTA---
gDCPAL4 ---TgcGATTTTAaT-G AATTgTTAATTTTA---
-849
-839
-837
-814

FIG. 3

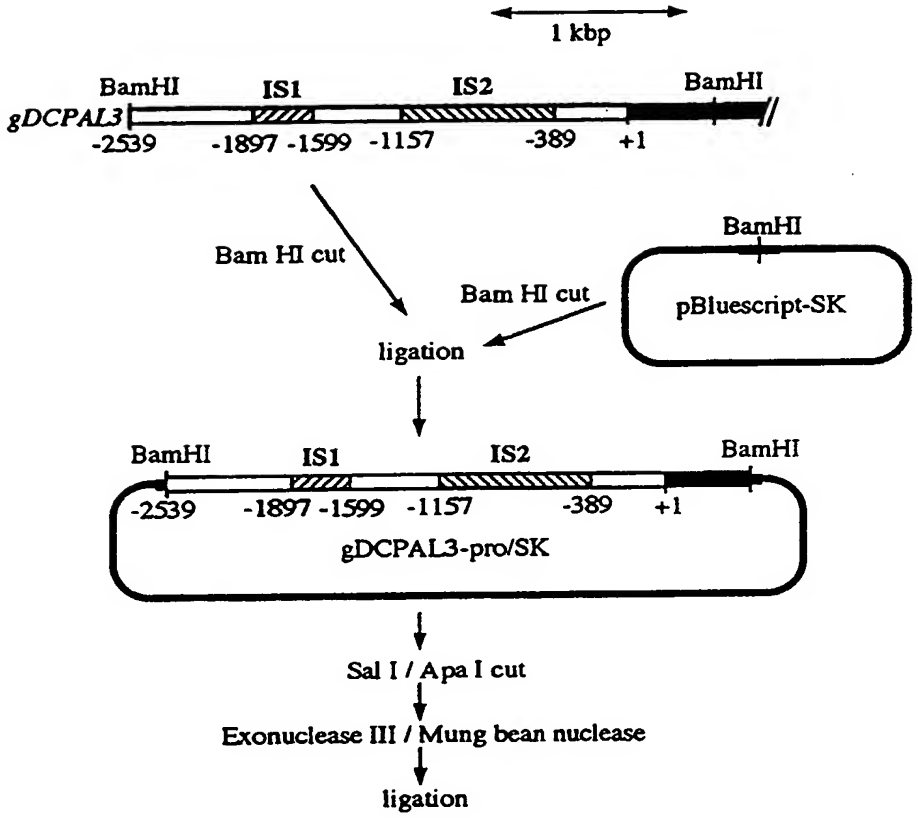

1 kbp
BamHI
IS1
IS2
BamHI
gDCPAL3
-2539
-1897
-1599
-1157
-389
+1
Bam HI cut
BamHI
Bam HI cut
pBluescript-SK
ligation
BamHI
IS1
IS2
BamHI
-2539
-1897
-1599
-1157
-389
+1
gDCPAL3-pro/SK
Sal I / Apa I cut
Exonuclease III / Mung bean nuclease
ligation

FIG. 4

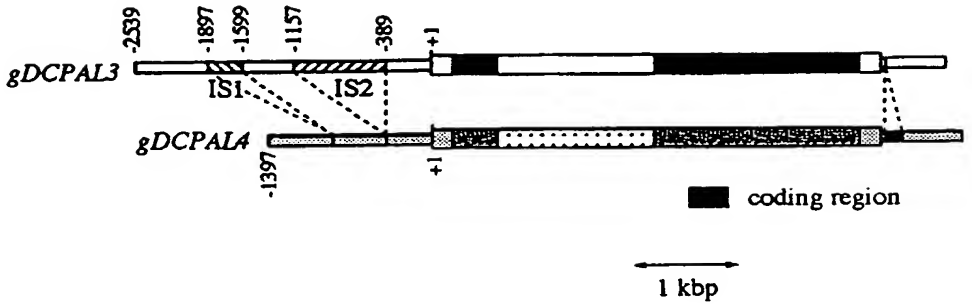

-2539
-1897
-1599
-1157
-389
+1
gDCPAL3
IS1
IS2
gDCPALA
-1397
+1
coding region
1 kbp

FIG. 5

Stowaway

FIG. 6

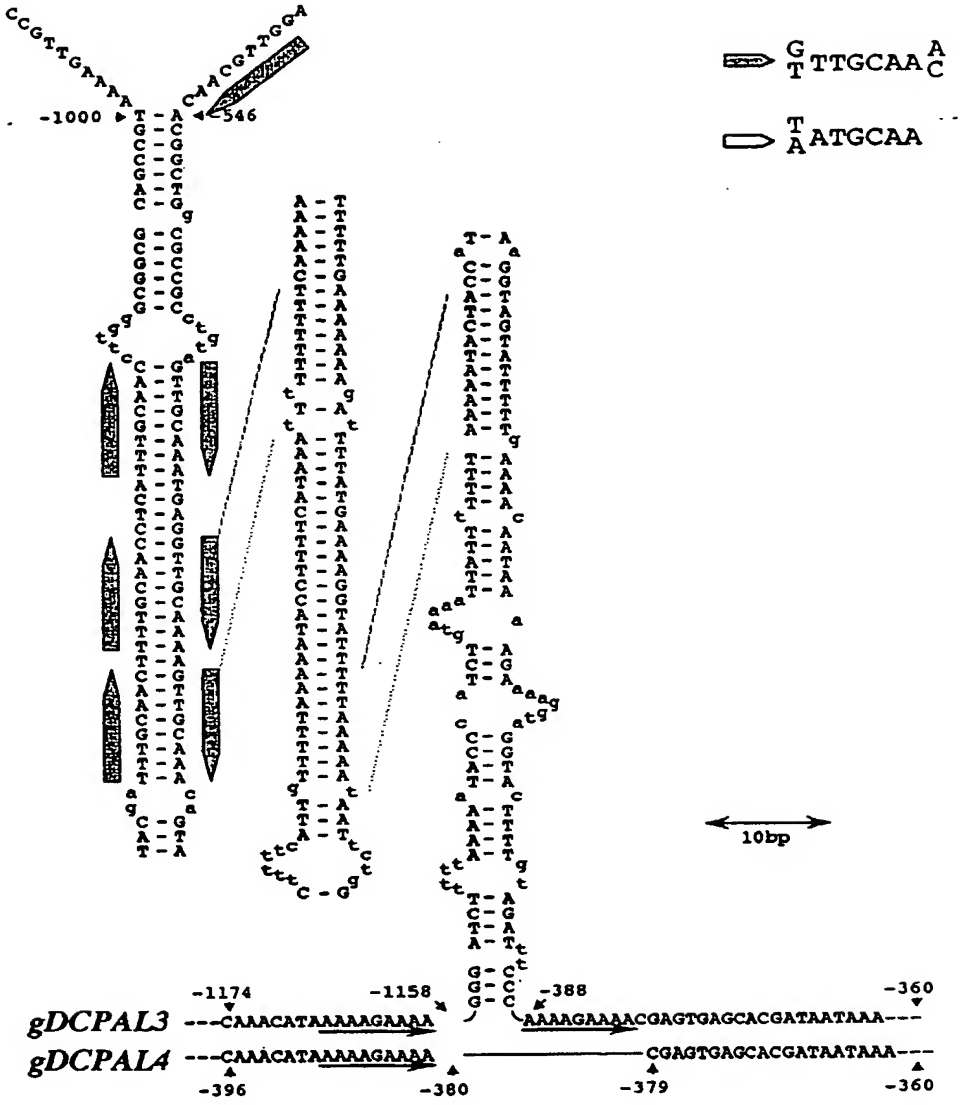

-1000
546
GTTTGCAAA
TATGCAA
10bp
-1174
-1158
-388
-360
gDCPAL3
gDCPAL4
-396
-380
-379
-360

FIG. 7

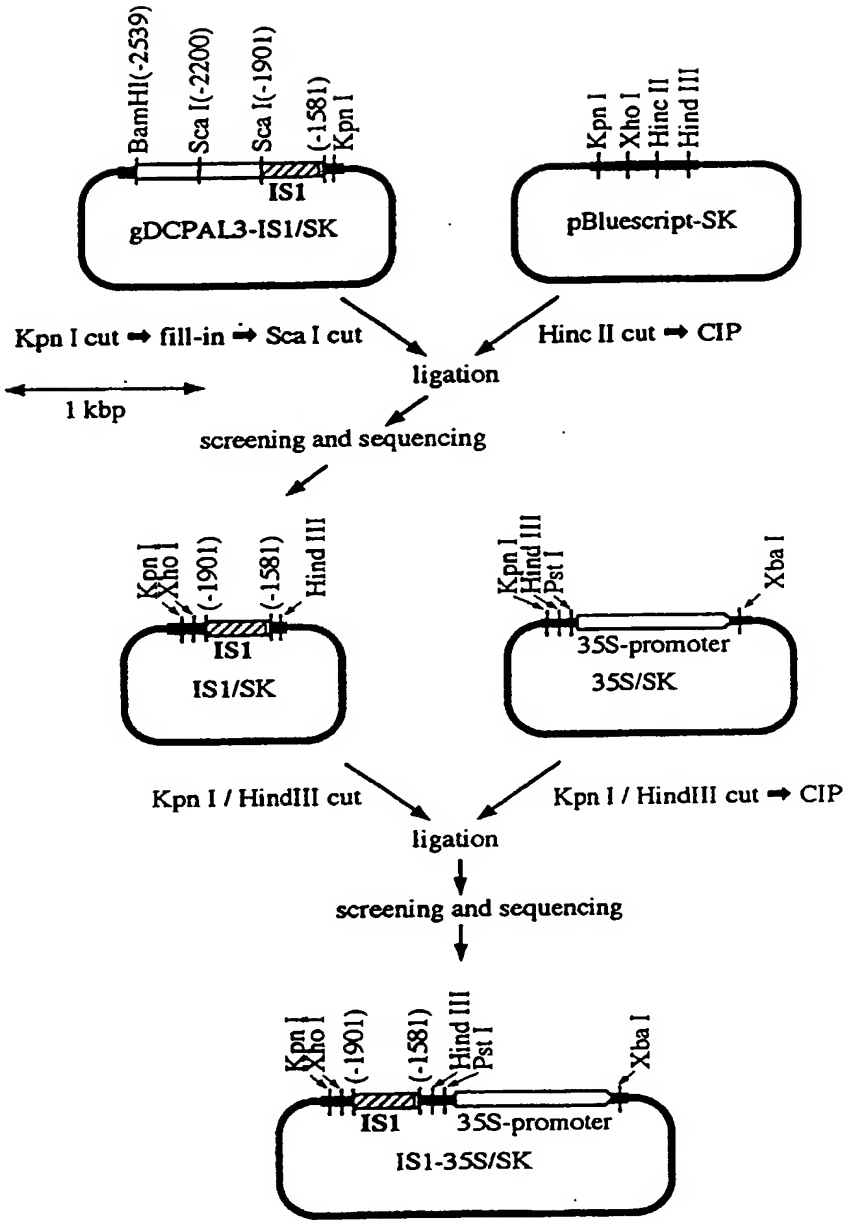

BamHI(-2539)
Sca I(-2200)
Sca I(-1901)
(-1581)
Kpn I
IS1
gDCPAL3-IS1/SK
Kpn I
Xho I
Hinc II
Hind III
pBluescript-SK
Kpn I cut ➡ fill-in ➡ Sca I cut
Hinc II cut ➡ CIP
ligation
1 kbp
screening and sequencing
Kpn I
Xho I
(-1901)
(-1581)
Hind III
IS1
IS1/SK
Kpn I
Hind III
Pst I
Xba I
35S-promoter
35S/SK
Kpn I / HindIII cut
Kpn I / HindIII cut ➡ CIP
ligation
screening and sequencing
Kpn I
Xho I
(-1901)
(-1581)
Hind III
Pst I
Xba I
IS1
35S-promoter
IS1-35S/SK

FIG. 8

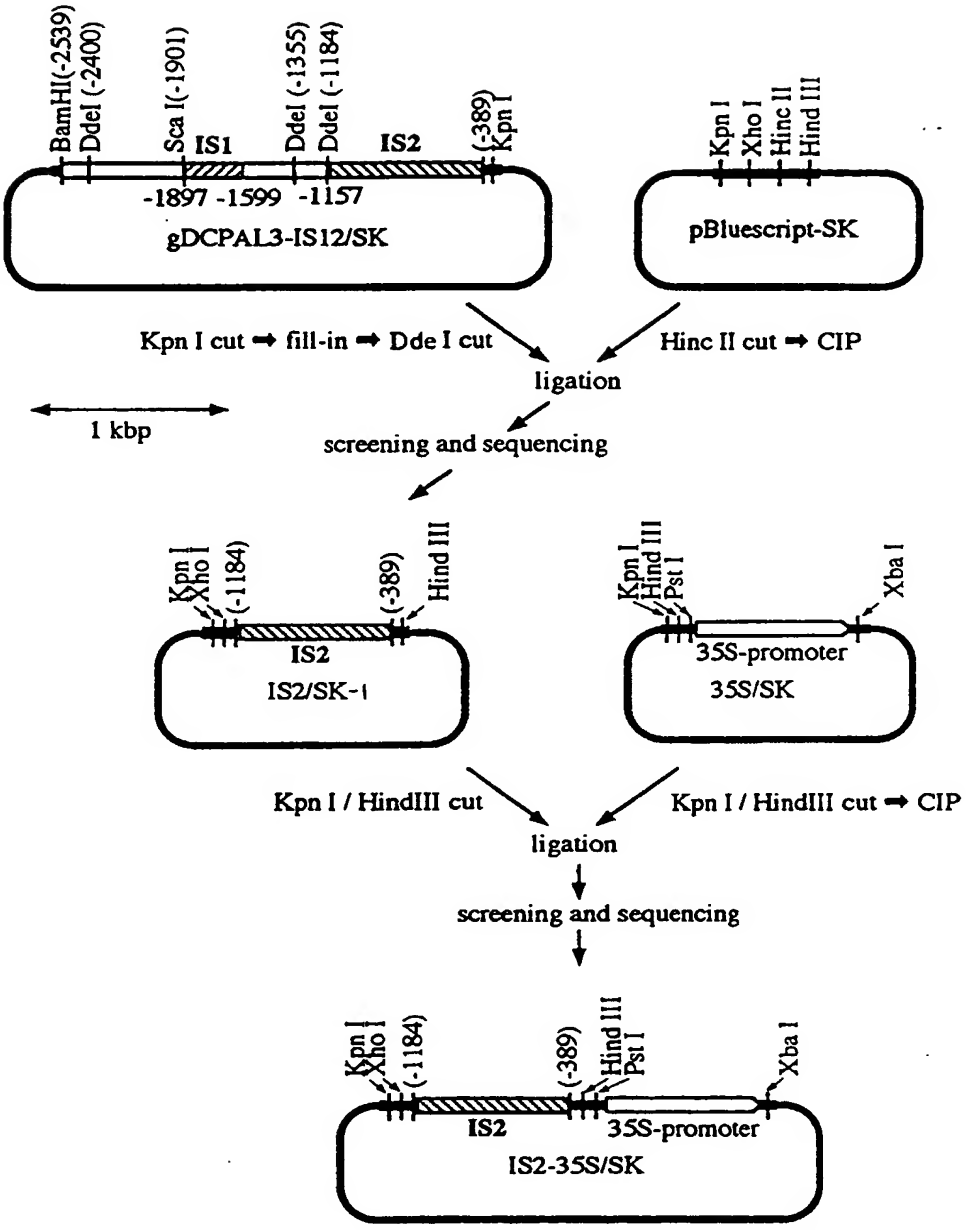

BamHI(-2539)
DdeI (-2400)
Sca I(-1901)
IS1
DdeI (-1355)
DdeI (-1184)
IS2
(-389)
Kpn I
-1897 -1599 -1157
gDCPAL3-IS12/SK
Kpn I
Xho I
Hinc II
Hind III
pBluescript-SK
Kpn I cut ➡ fill-in ➡ Dde I cut
Hinc II cut ➡ CIP
ligation
1 kbp
screening and sequencing
Kpn I
Xho I
(-1184)
(-389)
Hind III
IS2
IS2/SK-1
Kpn I
Hind III
Pst I
Xba I
35S-promoter
35S/SK
Kpn I / HindIII cut
Kpn I / HindIII cut ➡ CIP
ligation
screening and sequencing
Kpn I
Xho I
(-1184)
(-389)
Hind III
Pst I
Xba I
IS2
35S-promoter
IS2-35S/SK

FIG. 9

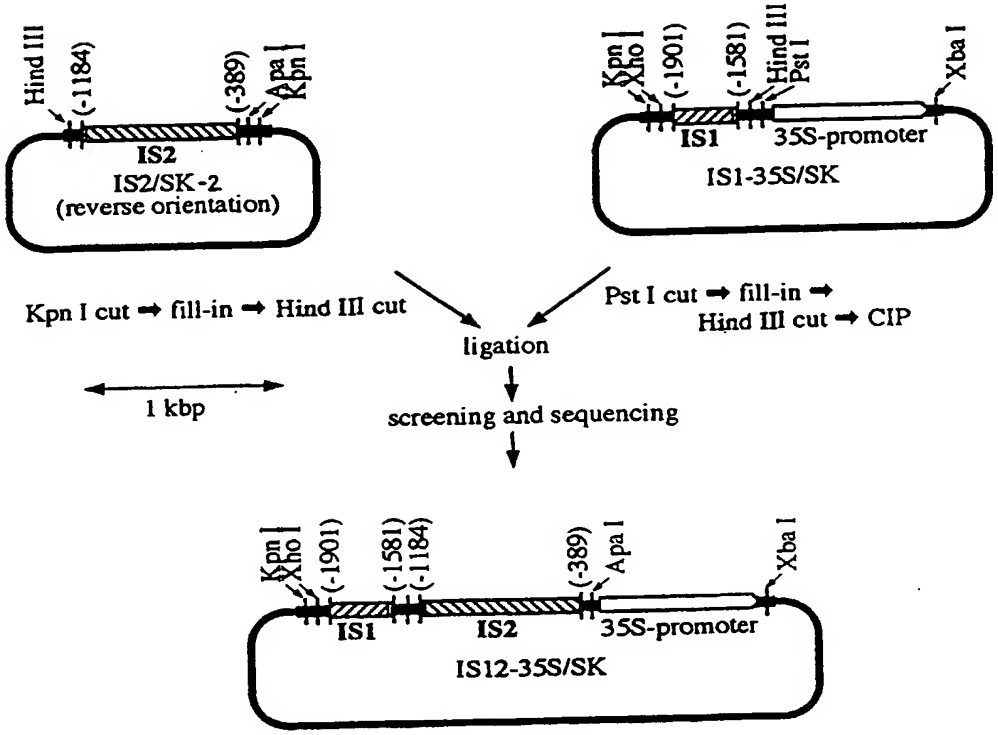

Hind III
(-1184)
(-389)
Apa I
Kpn I
IS2
IS2/SK-2
(reverse orientation)
Kpn I
Xho I
(-1901)
(-1581)
Hind III
Pst I
Xba I
IS1
35S-promoter
IS1-35S/SK
Kpn I cut ➡ fill-in ➡ Hind III cut
Pst I cut ➡ fill-in ➡
Hind III cut ➡ CIP
ligation
1 kbp
screening and sequencing
Kpn I
Xho I
(-1901)
(-1581)
(-1184)
(-389)
Apa I
Xba I
IS1
IS2
35S-promoter
IS12-35S/SK

FIG. 10

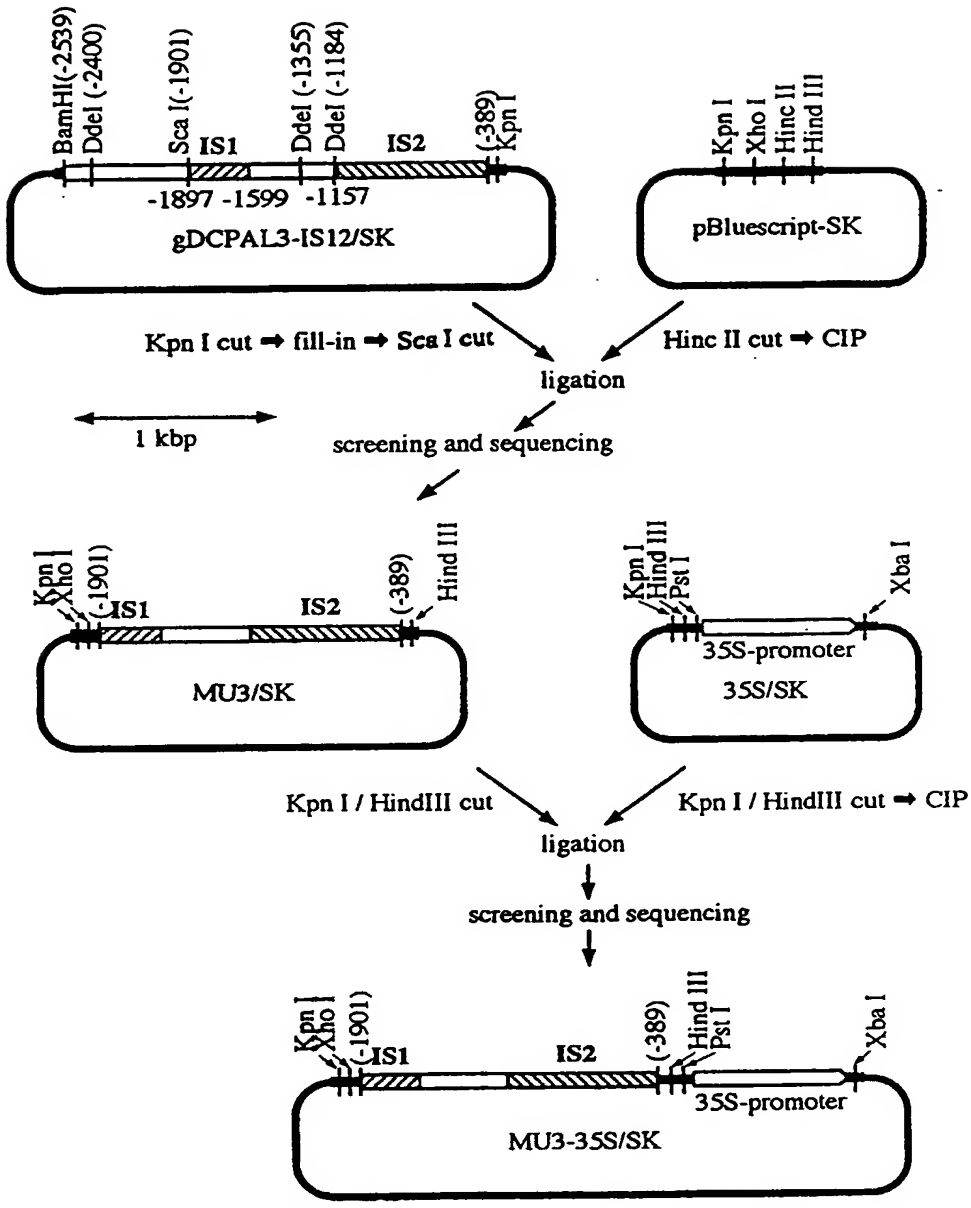

BamHI(-2539)
DdeI (-2400)
Sca I(-1901)
IS1
DdeI (-1355)
DdeI (-1184)
IS2
(-389)
Kpn I
-1897 -1599 -1157
gDCPAL3-IS12/SK
Kpn I
Xho I
Hinc II
Hind III
pBluescript-SK
Kpn I cut ➡ fill-in ➡ Sca I cut
Hinc II cut ➡ CIP
ligation
1 kbp
screening and sequencing
Kpn I
Xho I
(-1901)
IS1
IS2
(-389)
Hind III
MU3/SK
Kpn I
Hind III
Pst I
Xba I
35S-promoter
35S/SK
Kpn I / HindIII cut
Kpn I / HindIII cut ➡ CIP
ligation
screening and sequencing
Kpn I
Xho I
(-1901)
IS1
IS2
(-389)
Hind III
Pst I
Xba I
35S-promoter
MU3-35S/SK

FIG. 11

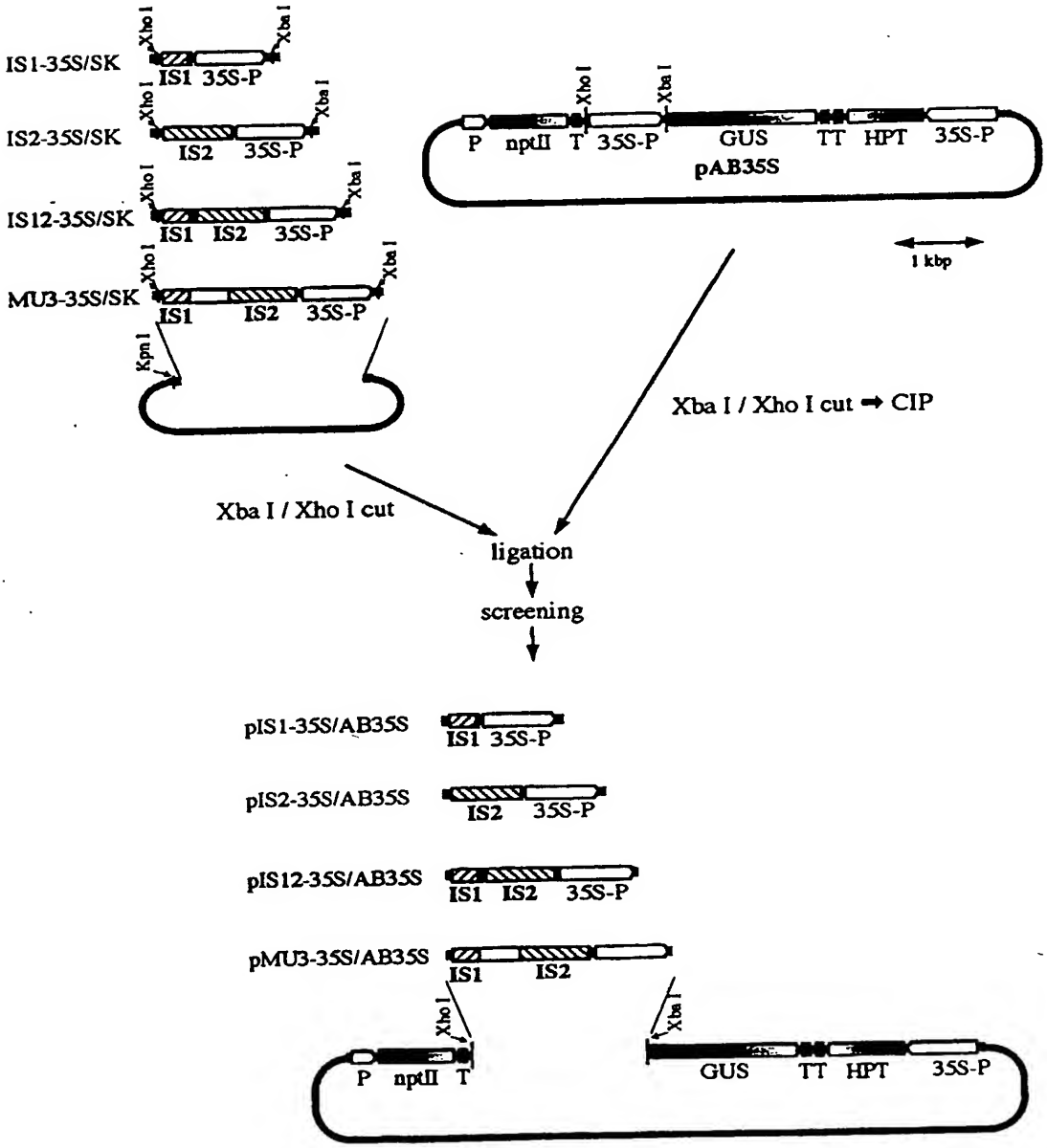

IS1-35S/SK
IS2-35S/SK
IS12-35S/SK
MU3-35S/SK
Xho I
Xba I
Kpn I
IS1 35S-P
IS2 35S-P
IS1 IS2 35S-P
P nptII T 35S-P GUS TT HPT 35S-P
pAB35S
1 kbp
Xba I / Xho I cut ➡ CIP
Xba I / Xho I cut
ligation
screening
pIS1-35S/AB35S
pIS2-35S/AB35S
pIS12-35S/AB35S
pMU3-35S/AB35S
IS1
IS2
P nptII T
GUS TT HPT 35S-P

FIG. 12

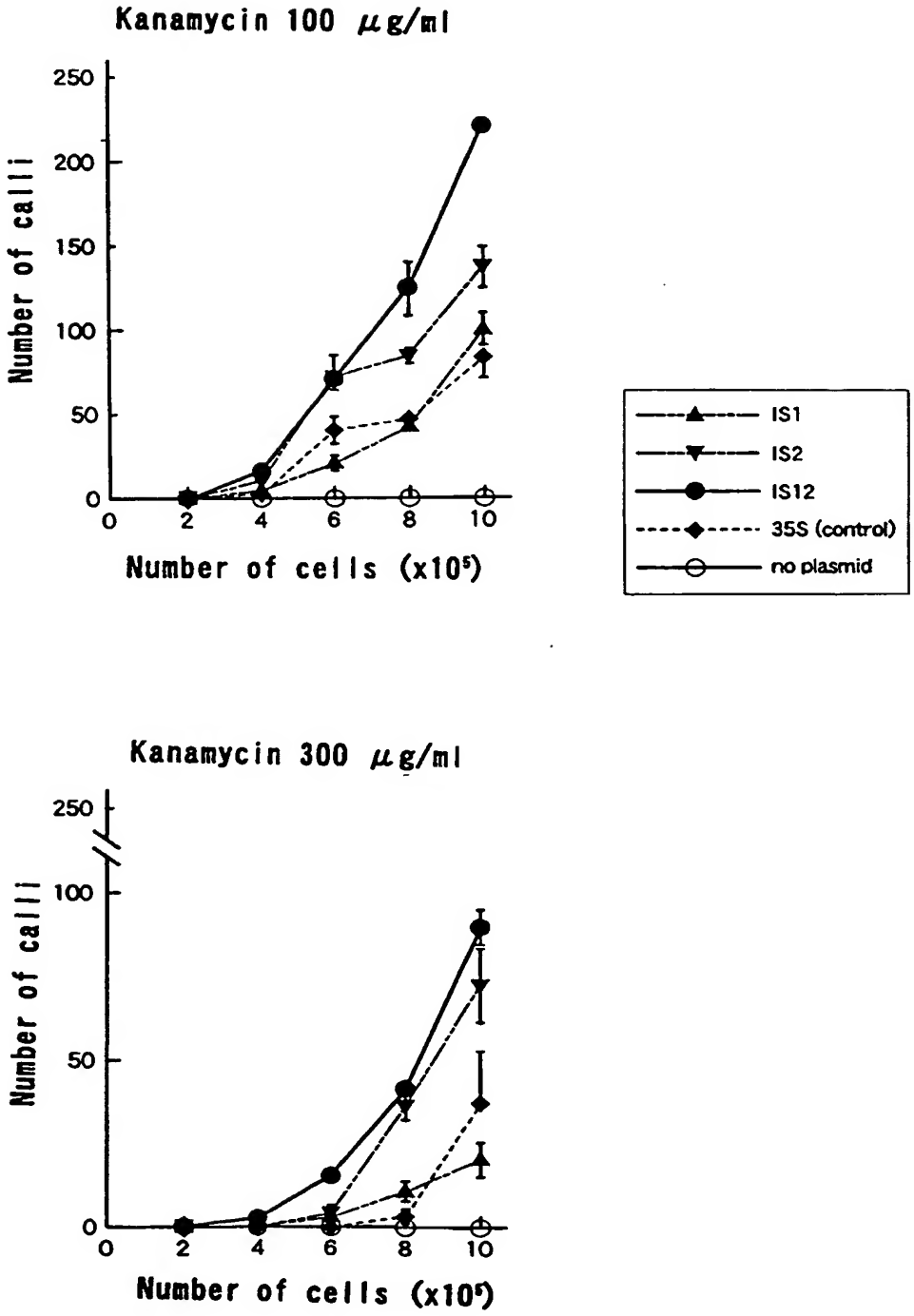
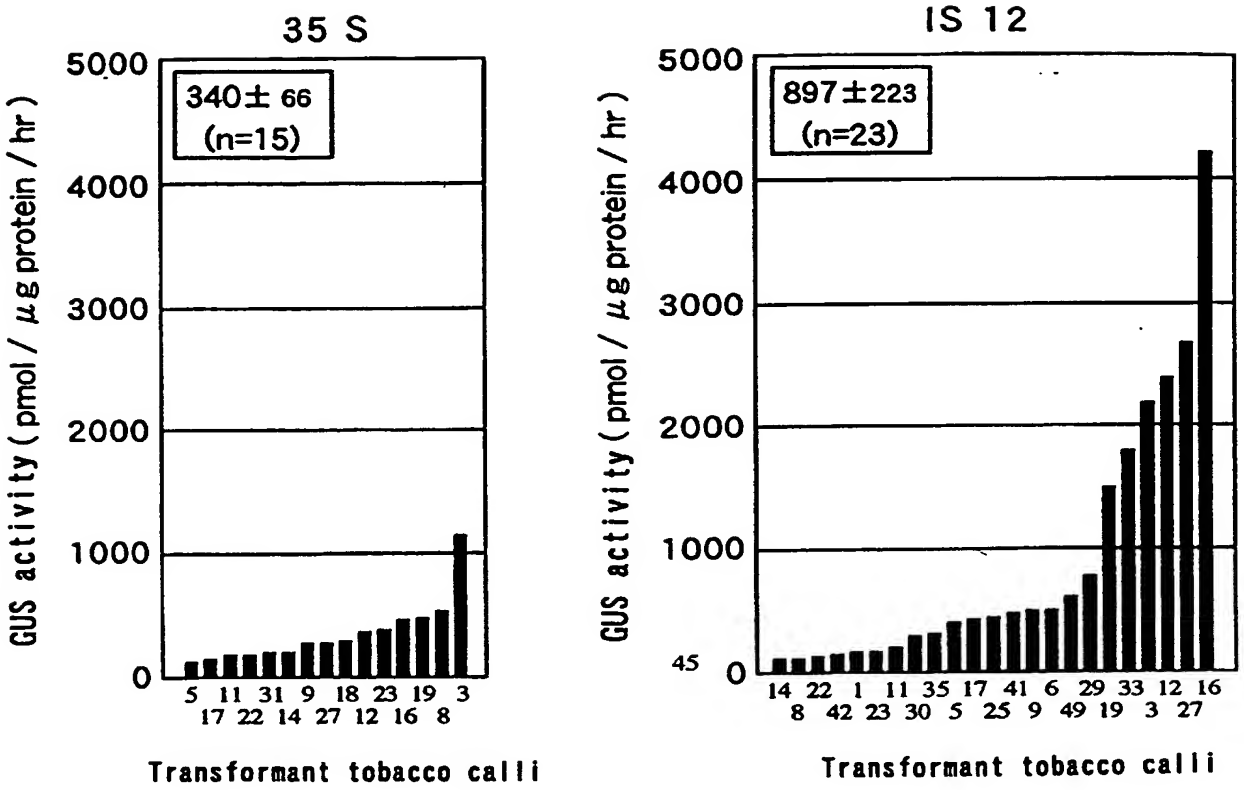

Kanamycin 100 μg/ml
Number of calli
250
200
150
100
50
0
0
2
4
6
8
10
Number of cells (x10⁵)
IS1
IS2
IS12
35S (control)
no plasmid
Kanamycin 300 μg/ml
Number of calli
250
100
50
0
0
2
4
6
8
10
Number of cells (x10⁵)

FIG. 13


35 S
340± 66
(n=15)
GUS activity (pmol / μg protein / hr)
5000
4000
3000
2000
1000
0
5 11 31 9 18 23 19 3
17 22 14 27 12 16 8
Transformant tobacco calli
IS 12
897±223
(n=23)
GUS activity (pmol / μg protein / hr)
5000
4000
3000
2000
1000
45
0
14 22 1 11 35 17 41 6 29 33 12 16
8 42 23 30 5 25 9 49 19 3 27
Transformant tobacco calli

SEQUENCE LISTING

<110> OZEKI, Yoshihiro

<110> SAN-EI GEN F.F.I.,INC.,

<120> NOVEL MINIATURE INVERTED-REPEAT TRANSPOSABLE ELEMENTS (MITEs)-LIKE ELEMENT AND TRANSCRIPTIONAL ACTIVATION ELEMENT

<130> P00-14

<150> JP 1999/206316

<151> 1999-07-21

<150> JP 1999/206320

<151> 1999-07-21

<150> JP 2000/175825

<151> 2000-06-12

<160> 14

<210> 1

<211> 769

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<213> Carrot (Daucus carota L.cv.Kurodagosun)

<400> 1

gggatctttt taaaaatacc catctgtaaa attatttttt taaaaatact accatctttt 60

tcattgtttt taaaaatacc ttttcataaa ttttttttttt caaaaatacg atttgcaact 120

tttgcaacct catttgcaac cttgggcggc gcagccgtaa aagttgccag tgaggttgca 180

aaagttgcaa atgagtttgt aaaagttgca aatgaggttg caaaagttgc aaataaaaat 240

ggaaagttgc aacagttgca actgcaattg caactagttc aactgaaaac tgtaagttgc 300

aaaagttgca aatgaggttg caactaaatg caactgaaaa ctgtaagtaa caacagatgt 360

atggtgtgcc cctggcgggg ccgttagatt acaatagaat caactgaatg caatcatatg 420

caactgaata caactatatg caatcatata tgcaattaca aatcctgatt tcaagttcca 480

gttttcgaat gtcattttcg aaatcgatat atatatatat atatatatat cgatttcgaa 540

aatgacattc gaaaactgga acttgaaatc aggaattcag ctgcatatga agttgcaaaa 600

gaggttgcaa cacggctggc gccgcctgta gttgcaaatg aggttgcaaa agttgcaaac 660

agtatttttg aaaaaaagat tttatgaaaa ggtattttta aaaataattc tggaaggtag 720

tatttttgaa aacaataaaa gaaaaggtag gtagttttgt agatttccc 769

<210> 2

<211> 299

<212> DNA

<213> Carrot (Daucus carota L.cv.Kurodagosun)

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cctagaatac ttactatttt ttaacactat ttttcactat tacacccacc aactctatat 120

tttatactat tttattatta aataaacact attacaccca ctacttttct ccactatctc 180

aaatctatta ttaaatattg ataggtccac cactttaccc acttttcaac tacatttact 240

acatttttct taatctccgt gaaagtcaaa ctcattcaca taaaatggga cagagggag 299

<210> 3

<211> 1192

<212> DNA

<213> Carrot (Daucus carota L.cv.Kurodagosun)

<400> 3

ggtaccgggc ccccctcga ggtcactccc tacgtcccat tttatgtgac ctcattttct 60

ttttgggacg tctcaaaaaa aataacctag aatacttact atttttaac actatttttc 120

actattacac ccaccaactc tatattttat actattttat tattaaataa acactattac 180

acccactact tttctccact atctcaaatc tattattaaa tattgatagg tccaccactt 240

tacccacttt tcaactacat ttactacatt tttcttaatc tccgtgaaag tcaaactcat 300

tcacataaaa tgggacagag ggagtaatta ttaattttaa tagacggtat cgataagctt 360

atcgataccg tctcagattc gcaaacataa aaagaaaagg gatcttttta aaaataccca 420

tctgtaaaat tatttttta aaaatactac catcttttc attgttttta aaaatacctt 480

ttcataaatt tttttttca aaaatacgat ttgcaacttt tgcaacctca tttgcaacct 540

tgggcggcgc agccgtaaaa gttgccagtg aggttgcaaa agttgcaaat gagtttgtaa 600

aagttgcaaa tgaggttgca aaagttgcaa ataaaaatgg aaagttgcaa cagttgcaac 660

tgcaattgca actagttcaa ctgaaaactg taagttgcaa aagttgcaaa tgaggttgca 720

actaaatgca actgaaaact gtaagtaaca acagatgtat ggtgtgcccc tggcggggcc 780

gttagattac aatagaatca actgaatgca atcatatgca actgaataca actatatgca 840

atcatatatg caattacaaa tcctgatttc aagttccagt tttcgaatgt cattttcgaa 900

atcgatatat atatatatat atatatatcg atttcgaaaa tgacattcga aaactggaac 960

ttgaaatcag gaattcagct gcatatgaag ttgcaaaaga ggttgcaaca cggctggcgc 1020

cgcctgtagt tgcaaatgag gttgcaaaag ttgcaaacag tatttttgaa aaaaagattt 1080

tatgaaaagg tatttttaaa aataattctg gaaggtagta tttttgaaaa caataaaaga 1140

aaaggtaggt agttttgtag atttcccaga cctcgagggg gggcccggta cc 1192

<210> 4

<211> 8

<212> DNA

<213> Carrot (Daucus carota L.cv.Kurodagosun)

<400> 4

gttgcaaa 8

<210> 5

<211> 8

<212> DNA

<213> Carrot (Daucus carota L.cv.Kurodagosun)

<400> 5

gttgcaac 8

<210> 6

<211> 8

<212> DNA

<213> Carrot (Daucus carota L.cv.Kurodagosun)

<400> 6

tttgcaaa 8

<210> 7

<211> 8

<212> DNA

<213> Carrot (Daucus carota L.cv.Kurodagosun)

<400> 7

tttgcaac 8

<210> 8

<211> 7

<212> DNA

<213> Carrot (Daucus carota L.cv.Kurodagosun)

<400> 8

tatgcaa 7

<210> 9

<211> 7

<212> DNA

<213> Carrot (Daucus carota L.cv.Kurodagosun)

<400> 9

aatgcaa 7

<210> 10

<211> 158

<212> DNA

<213> Carrot (Daucus carota L.cv.Kurodagosun)

<400> 10

gggatctttt taaaaatacc catctgtaaa attatttttt taaaaatact accatctttt 60

tcattgtttt taaaaatacc ttttcataaa ttttttttt caaaaatacg atttgcaact 120

tttgcaacct catttgcaac cttgggcggc gcagccgt 158

<210> 11

<211> 158

<212> DNA

<213> Carr t (Daucus carota L.cv.Kurodagosun)

<400> 11

acggctggcg ccgcctgtag ttgcaaatga ggttgcaaaa gttgcaaaca gtatttttga 60

aaaaaagatt ttatgaaaag gtatttttaa aaataattct ggaaggtagt atttttgaaa 120

acaataaaag aaaaggtagg tagttttgta gatttccc 158

<210> 12

<211> 32

<212> DNA

<213> Carrot (Daucus carota L.cv.Kurodagosun)

<400> 12

ctccctacgt cccattttat gtgacctcat tt 32

<210> 13

<211> 32

<212> DNA

<213> Carrot (Daucus carota L.cv.Kurodagosun)

<400> 13

aaactcattc acataaaatg ggacagaggg ag 32

<210> 14

<211> 1553

<212> DNA

<213> Carrot (Daucus carota L.cv.Kurodagosun)

<400> 14

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ttttgggacg tctcaaaaaa aataacctag aatacttact atttttaac actatttttc 120

actattacac ccaccaactc tatattttat actattttat tattaaataa acactattac 180

acccactact tttctccact atctcaaatc tattattaaa tattgatagg tccaccactt 240

tacccacttt tcaactacat ttactacatt tttcttaatc tccgtgaaag tcaaactcat 300

tcacataaaa tgggacagag ggagtaatta ttaattttaa taaattatat gtgattttga 360

ttatgtgtgg attcttgata aaatatcaca gagttgatat aaattctaaa gatttaacca 420

caatgtttca aaatctcata gattttagta ggattcaaaa aatttaaaat acgttcaaaa 480

atctcataaa attcatcatt ttattaaatc caaaaaaatc cagtaatatt tgacaatcag 540

attttaatga attttaaatt gaacaatctc agttgaatac catgagattt taaaatataa 600

tttaaaattc taattgaata ccaccagatt ttgtaaaata atttaaaatt ttaattgaat 660

attcaagatt ttaatatatt ttaaataatc tcgatctgaa ttacaaaaaa tgcttaaaat 720

ctgaatccca tacgtcctct cagattcgca aacataaaaa gaaaagggat ctttttaaaa 780

atacccatct gtaaaattat ttttttaaaa atactaccat ctttttcatt gtttttaaaa 840

atacctttc ataaattttt tttttcaaaa atacgatttg caacttttgc aacctcattt 900

gcaaccttgg gcggcgcagc cgtaaaagtt gccagtgagg ttgcaaaagt tgcaaatgag 960

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atatgcaatc atatatgcaa ttacaaatcc tgatttcaag ttccagtttt cgaatgtcat 1260

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aagattttat gaaaaggtat ttttaaaaat aattctggaa ggtagtattt ttgaaaacaa 1500

taaaagaaaa ggtaggtagt tttgtagatt tcccagacgg tatcgataag ctt 1553

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(71) Applicant *(for all designated States except US)*: **SAN-EI GEN F.F.I., INC.** [JP/JP]; 1-11, Sanwa-cho 1-chome, Toyonaka-shi, Osaka 561-0828 (JP).

(71) Applicant and
(72) Inventor: **OZEKI, Yoshihiro** [JP/JP]; 2-3-6-302, Daimon-cho, Higashikurume-shi, Tokyo 203-0011 (JP).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **OYANAGI, Mikiko** [JP/JP]; 2-6-1-1007, Midori-cho, Fuchu-shi, Tokyo 183-0006 (JP). **FUKUDA, Takashi** [JP/JP]; 612-8, Kamimizo, Sagamihara-shi, Kanagawa 229-1123 (JP). **KODA, Takatoshi** [JP/JP]; San-Ei Gen F.F.I., Inc., 1-11, Sanwa-cho 1-chome, Toyonaka-shi, Osaka 561-0828 (JP).

(74) Agents: **SAEGUSA, Eiji** et al.; Kitahama TNK Building, 1-7-1, Doshomachi, Chuo-ku, Osaka-shi, Osaka 541-0045 (JP).

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(54) Title: MITES-LIKE ELEMENT AND TRANSCRIPTIONAL ACTIVATION ELEMENT

(57) Abstract: The invention provides novel, carrot-derived MITE-like elements (transposable elements). It further provides transcriptional activation elements comprising at least one transposable element, in particular one of the above MITE-like elements. Specifically, it provides transcriptional activation elements having a DNA comprising the nucleotide sequence shown under SEQ ID NO:1 or a functional equivalent thereto and/or a DNA comprising the nucleotide sequence shown under SEQ ID NO:2 or a functional equivalent thereto. The transcriptional activation elements of the invention can increase or activate the reduced expression of a foreign gene introduced by the transgenic technology. Therefore, the transcriptional activation elements contribute to stable expression of a foreign gene introduced in a plant genome and are useful in stably producing genetically modified plants.

INTERNATIONAL SEARCH REPORT

Inte[redacted]ional Application No
[redacted]JP 00/04837

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/90 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBL, WPI Data, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BUREAU T E ET AL: "Stowaway: a new family of inverted repeat elements associated with the genes of both monocotyledonous and dicotyledonous plants" PLANT CELL,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD,US, vol. 6, no. 6, June 1994 (1994-06), pages 907-916, XP002156106 ISSN: 1040-4651 cited in the application the whole document --- -/--	1-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BÉNÉDICTE CHARRIER ET AL.: "Bigfoot: a new family of MITE elements characterized from the Medicago genus" PLANT JOURNAL., vol. 18, no. 4, May 1999 (1999-05), pages 431-441, XP002157570 BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD., GB ISSN: 0960-7412 the whole document	1-5,9-19
A	ELENA CASACUBERTA ET AL.: "Presence of miniature inverted-repeat transposable elements (MITEs) in the genome of Arabidopsis thaliana: characterisation of the Emigrant family of elements" PLANT JOURNAL., vol. 16, no. 1, October 1998 (1998-10), pages 79-85, XP002156103 BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD., GB ISSN: 0960-7412 the whole document	1-5,9-19
X	WO 98 22593 A (PIONEER HI-BRED INTERNATIONAL, INC.) 28 May 1998 (1998-05-28) page 6, line 27 -page 7, line 17 page 7, line 31 -page 8, line 7; examples 1,2	8,13-19
X	SHIN TAKEDA ET AL.: "Transcriptional activation of the tobacco retrotransposon Ttol by wounding and methyl jasmonate" PLANT MOLECULAR BIOLOGY, vol. 36, 1998, pages 365-376, XP002165578 abstract page 370, left-hand column, paragraph 1 -right-hand column, paragraph 2 page 373, right-hand column, paragraph 2 -page 374, right-hand column, paragraph 2	8,13-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP 00/04837

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-5, 10-12, and partially 8, 9 and 13-19

   MITE-like element capable of causing duplication of the target sequence (A)nG(A)n; transcriptional activation element containing it; transgene expression cassette and plasmid containing the same and transgenic plant containing such an expression cassette

2. Claims: 6 and 7, and partially 8, 9 and 13-19

   MITE-like element capable of causing duplication of the target sequence TA, comprising SEQ ID NO:6 and showing SEQ ID NO:4 in the 5' terminal region and SEQ ID NO:5 in the 3' terminal region; transcriptional activation element containing it; transgene expression cassette and plasmid containing the same and transgenic plant containing such an expression cassette

3. Claims: partially 8, 9 and 13-19

   Transcriptional activation element containing a transposable element other than a MITE-like element under subject 1 and 2; transgene expression cassette and plasmid containing the same and transgenic plant containing such an expression cassette

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
JP 00/04837

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9822593 A	28-05-1998	US 5955361 A	21-09-1999
		AU 5455498 A	10-06-1998